719,045 AT

REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978
APPLICATION FOR A PATENT AND
ACKNOWLEDGEMENT OF RECEIPT
(Section 30(1) Regulation 22)

FORM P.1
(to be lodged in duplicate)

R 8,00 for Provisional
R36,00 for Complete

THE GRANT OF A PATENT IS HEREBY REQUESTED BY THE UNDERMENTIONED APPLICANT ON THE BASIS OF THE PRESENT APPLICATION FILED IN DUPLICATE.

PATENT APPLICATION NO.		
21	01	858794
71	FULL NAME(S) OF APPLICANT(S)	

A & A REF: 110652

AMERICAN CYANAMID COMPANY

ADDRESS(ES) OF APPLICANT(S)

One Cyanamid Plaza, Wayne, State of New Jersey 07470, USA

54 TITLE OF INVENTION

ANTITUMOR ANTIBIOTICS (LL-E33288 COMPLEX)

Only the items marked with an "X" in the blocks below are applicable.

- [x] THE APPLICANT CLAIMS PRIORITY AS SET OUT ON THE ACCOMPANYING FORM P.2
- [ ] THE APPLICATION IS FOR A PATENT OF ADDITION TO PATENT APPLICATION NO. 21 | 01
- [ ] THIS APPLICATION IS A FRESH APPLICATION IN TERMS OF SECTION 37 AND BASED ON APPLICATION NO. 21 | 01

THIS APPLICATION IS ACCOMPANIED BY:

- [x] 1. ~~A single copy of a provisional or~~ two copies of a complete specification of ....58..... pages
- [x] 2. Drawings of .18..... sheets. INFORMAL
- [x] 3. Publication particulars and abstract (Form P.8 in duplicate) (for complete only).
- [ ] 4. A copy of Figure .... of the drawings (if any) for the abstract (for complete only).
- [ ] 5. An assignment of invention.
- [x] 6. Certified priority document(s) (State quantity) : ..1........
- [ ] 7. Translation of the priority document(s).
- [ ] 8. An assignment of priority rights.
- [ ] 9. A copy of the Form P.2 and the specification of S.A. Patent Application No. 21 | 01
- [x] 10. A Form P.2 in duplicate.
- [x] 11. A declaration and power of attorney on Form P.3.
- [ ] 12. Request for ante-dating on Form P.4.
- [ ] 13. Request for classification on Form P.9.
- [ ] 14. Request for delay of acceptance on Form P.4.
- [ ] 15.

74 ADDRESS FOR SERVICE: Adams & Adams, Pretoria.

DATED THIS 15 DAY OF NOVEMBER 19 85

J.R. Steyn
ADAMS & ADAMS
APPLICANTS PATENT ATTORNEYS

The duplicate will be returned to the applicant's address for service as proof of lodging but is not valid unless endorsed with official stamp.

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FORM P7

REPUBLIC OF SOUTH AFRICA

Patents Act, 1978

COMPLETE SPECIFICATION

(Section 30 (1) — Regulation 28)

OFFICIAL APPLICATION NO.		
21	01	858794

LODGING DATE	
22	15 November 1985

INTERNATIONAL CLASSIFICATION	
51	A61K C07G C12P C12N C12R

FULL NAME(S) OF APPLICANT(S)

71

AMERICAN CYANAMID COMPANY

FULL NAME(S) OF INVENTOR(S)

72

MAY DEAN-MING LEE
MICHAEL GREENSTEIN
DAVID PAUL LABEDA

TITLE OF INVENTION

54

ANTITUMOR ANTIBIOTICS (LL-E33288 COMPLEX)

[redacted]

Title: ANTITUMOR ANTIBIOTICS
(LL-E33288 COMPLEX)

SUMMARY OF THE INVENTION

This application is a continuation-in-part of co-pending application S.N. 672,031, field November 16, 1984.

This invention relates to new antibacterial and antitumor agents designated LL-E33288α_1-Br, LL-E33288 α_1-I LL-E33288α_2-Br, LL-E33288α_2-I, LL-E33288α_3-Br, LL-E33288α_3-I, LL-E33288α_4-Br, LL-E33288β_1-Br, LL-E33288β_1-I, LL-E33288 β_2-Br, LL-E33288β_2-I, LL-E33288 γ_1-Br, LL-E33288γ_1-I and LL-E33288δ_1-I, to their production by fermentation, to methods for their recovery and concentration from crude solutions and to processes for their purification. The present invention includes within its scope the antibacterial and antitumor agents in dilute form, as crude concentrates, as a complex of various or all components, in pure form as individual components and novel strains of _Micromonospora_.

The LL-E33288 antibiotics of this invention are closely related compounds. The fourteen antibiotics are recovered from fermentation and are initially obtained as a mixture, hereinafter either the LL-E33888 complex, the the LL-E33288 Iodo-complex or the LL-E33288 Bromo-complex. In general, the iodine containing components of the LL-E33288 antibiotics (_e.g._, α_1-I, α_2-I, α_3-I, β_1-I, β_2-I, γ_1-I and δ_1-I) are found only in fermentations using media containing inorganic or organic iodide while the bromine containing components (_e.g._, α_1-Br, α_2-Br, α_3-Br, α_4-Br, β_1-Br, β_2-Br and γ_1- Br) are found only in fermentations using media containing inorganic or organic bromide. The ratio of components in the LL-E3388 complex will vary, depending upon

the fermentation of both the bromine and the iodine containing antibiotics, LL-E33288β_1 and LL-E33288γ_1 are the major components, together accounting for approximately 90% of the complex. LL-E33288α_1, LL-E33288α_2, LL-E33288α_3, LL-E33288α_4-Br, LL-E33288β_2 and LL-E33288δ_1-I are minor components, together accounting for approximately 10% of the complex.

The LL-E33288 antibiotics are active against gram-positive and gram-negative bacteria. Each of the components were also found to be active in a modification of the Biochemical Induction Assay [Elespuru, R. and Yarmolinsky, M., Environmental Mutagenesis, 1, 65-78 1979)]. a test which specifically measures the ability of an agent to directly or indirectly initiate DNA damage. In this assay, both LL-E33288β_1-Br and LL-E33288γ_1-Br were active at concentrations lower than $1X10^{-6}$ mcg/ml.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. I is the ultraviolet absorption spectra of LL-E33288β_1-Br;

FIG. II is the infrared absorption spectrum of LL-E33288β_1-Br;

FIG. III is the proton magnetic resonance spectrum of LL-E33288β_1-Br;

FIG. IV is the carbon 13 magnetic resonance spectrum of LL-E33288 β_1-Br;

FIG. V is the ultraviolet absorption spectra of LL-E33288γ_1-Br;

FIG. VI is the infrared absorption spectrum of LL-E33288γ_1-Br;

FIG. VII is the proton magnetic resonance spectrum of LL-E33288 γ_1-Br;

FIG. VIII is the carbon 13 magnetic resonance spectrum of LL-E33288γ_1-Br;

FIG. IX is the proton magnetic resonance spectrum of LL-E33288 α_2-I;

FIG. X is the proton magnetic resonance spectrum of LL-E33288 α_3-I;

FIG. XI is the ultraviolet absorption spectra of LL-E33288β_1-I;

FIG. XII is the infrared absorption spectrum of LL-E33288β_1-I;

FIG. XIII is the protein magnetic resonance spectrum of LL-E33288β_1-I;

FIG. XIV is the carbon 13 magnetic resonance spectrum of LL-E33288β_1-I;

FIG. XV is the ultraviolet absorption spectra of LL-E33288γ_1-I;

FIG. XVI is the infrared absorption spectrum of LL-E33288γ_1-I;

FIG. XVII is the proton magnetic resonance spectrum of LL-E33288γ_1-I; and

FIG. XVIII is the carbon 13 magnetic resonance spectrum of LL-E33288γ_1-I.

DETAILED DESCRIPTION OF THE INVENTION

The physico-chemical characteristics of LL- E33288 β_1-Br and LL-E33288γ_1-Br are described below:

LL-E33288β_1-Br

1) Approximate elemental analysis: C 48.6; H 5.6; N 2.9; S 9.1; and Br 5.5. (It has been determined by electron spectroscopy for chemical analysis (ESCA) that only the following elements are present: C, H, N, O, S and Br);
2) Melting point: 146-150°C (dec.);
3) Specific rotation: $[\alpha]_D^{26} = -49\pm10°$ (0.1% ethanol);
4) Ultraviolet absorption spectra: as shown in Figure I (methanol; acidic methanol; basic methanol);
5) Infrared absorption spectrum: as shown in Figure II (KBr disc);
6) Proton magnetic resonance spectrum: as shown in Figure III (300 MHz, $CDCl_3$);
7) Carbon-13 magnetic resonance spectrum: as shown in Figure IV (75.43 MHz, $CDCl_3$, ppm from TMS), significant peaks as listed below:

17.60(q);	17.64(q);	18.9(q);	19.7(q);
22.4(q);	22.8(q);	23.5(q);	34.3(t):
36.9(t);	39.2 (t/d);	47.8 (d);	51.7(q);
52.7(q);	54.6 (t/d);	56.3(q);	57.2(q);
57.8(d);	61.0 (q/d);	61.7(d);	62.4(t);
66.9(d);	68.4(d);	69.1(d);	69.7(d);
70.2(d);	71.1(d);	71.9(d);	72.1(s/t);
76.1(d);	81.0(d);	83.3(s);	88.2(s);
97.4(d);	99.7(d);	100.8(s);	102.5(d);
115.1(s);	123.4(d);	124.4(d);	126.5(d);
130.2(s):	130.8(s);	144.6(s):	149.3(s);
149.5(s):	191.7(s);	192.4(s);	

8) Molecular weight: 1333/1335 respectively for $^{79}Br/^{81}Br$ as determined by FAB-MS; and

9) Molecular formula: $C_{54}H_{84}N_3O_{22}S_4Br$, exact masses at 1258.3699 (^{79}Br) and 1260.3726(^{81}Br) was determined by high resolution FAB-MS and calculated to be $C_{52}H_{81}N_3O_{21}S_3Br$ (M+H-C_2H_4OS).

<u>LL-E33288 γ_1-Br</u>

1) Ultraviolet absorption spectra: as shown in Figure V (methanol; acidic methanol; basic methanol);

2) Infrared absorption spectrum: as shown in Figure VI (KBr disc):

3) Proton magnetic resonance spectrum: as shown in Figure VII (300 MHz, $CDCl_3$);

4) Carbon 13 magnetic resonance spectrum: as shown in Figure VIII (75.43 MHZ, $CDCl_3$, ppm from TMS), significant peaks as listed below:

14.4	17.6	17.9	19.0
19.7	-	22.8	-
-	34.0	37.6	39.5
42.1	-	51.6	52.7
54.1	56.3	57.3	-
59.3	61.1	61.8	61.9
67.2	68.18	68.23	69.7
70.1	70.8	71.1	71.7
71.8	76.1	-	81.0
82.9	88.4	-	97.8
100.0	100.2	101.3	103.0
115.3	123.0	124.9	126.9
130.4	131.1	131.8	138.0
144.7	-	149.5	149.6
155.6	192.5	192.9	

5) Molecular formula: $C_{53}H_{82}N_3O_{22}S_4Br$ by comparing its UV, IR, 'H NBR, and ^{13}C NMR data to those of LL-E33288 β_1-BR and LL-E33288 γ_1-I; and

6) Molecular weight: 1319/1321 respectively for $^{79}Br/^{81}Br$, calculated from its molecular formula.

The physico-chemical characteristics of LL-E33288 α_1-I, LL-E33288 α_2-I, LL-E33288 α_3-I, and LL-E33288 β_1-I and LL-E33288 γ_1-I are described below:

<u>LL-E33288 α_1-I</u>

1) Molecular weight: 1145, determined by FAB-MS.

<u>LL-E33288 α_2-I</u>

1) Contains and only contains the following elements by electron spectroscopy for chemical analysis (ESCA): C, H, N, O, S, I;
2) Molecular weight: 1131, determined by FAB-MS; and
3) Proton magnetic resonance spectrum: as shown in Figure IX (300 MHz, $CDCl_3$).

<u>LL-E33288 α_3-I</u>

1) Molecular weight: 1066, determined by FAB-MS; and
2) Proton magnetic resonance spectrum: as shown in

Figure X (300 MHz, $CDCl_3$).

LL-E33288 β_1-I

1) Ultraviolet absorption spectra: as shown in Figure XI (methanol; acidic methanol; basic methanol);
2) Infrared absorption spectrum: as shown in Figure XII (KBr disc);
3) Proton magnetic resonance spectrum: as shown in Figure XIII (300 MHz, $CDCl_3$);
4) Carbon 13 magnetic resonance spectrum: as shown in Figure XIV (75.43 MHz, $CDCl_3$, ppm from TMS), significant peaks as listed below:

-	17.5	17.6	18.9
-	22.4	22.8	23.4
25.4	34.3	36.9	39.2
-	47.9	51.6	52.8
54.8	56.3	57.2	57.9
60.9		61.6	62.2
67.0	68.4	68.4	69.1
69.6	70.4	71.1	71.8
72.2	76.2	-	80.8
83.3	88.1	93.6	97.4
99.6	99.6	-	102.6
112.4	123.4	124.4	126.4
-	-	133.4	-
-	-	-	-
-	192.3	192.6	

5) Molecular formula: $C_{54}H_{84}N_3O_{22}S_4I$ by comparing its UV, IR, ^1H NMR and ^{13}C NMR data to those of LL-E33288 β_1-Br and LL-E33288 γ_1-I; and
6) Molecular weight: 1381, calculated from molecular formula.

LL-E33288 γ_1-I

1) Contains and only contains the following elements by electron spectroscopy for chemical analysis (ESCA): C,H,N,O,S,I;
2) Approximate elemental analysis: C 48.8; H 5.4; N 2.8; S 9.0; I 9.2;

3) Molecular weight: 1367, determined by FAB-MS;

4) Molecular formula: $C_{53}H_{82}N_3O_{22}S_4I$, exact mass for M+H was determined by high resolution FAB-MS to be 1368. 3397 for $C_{53}H_{83}N_3O_{22}S_4I$;

5) Ultraviolet absorption spectra: as shown in Figure XV (methanol; acidic methanol; basic methanol);

6) Infrared absorption spectrum: as shown in Figure XVI (KBr disc);

7) Proton magnetic resonance spectrum: as shown in Figure XVII (300 MHz, $CDCl_3$); and

8) Carbon 13 magnetic resonance spectrum: as shown in Figure XVIII (75.43 MHz, $CDCl_3$, ppm for TMS) significant peaks as listed below:

14.5(q)	17.6(q)	17.6(q)	18.9(q)
-	-	22.8(q)	-
25.4(q)	34.1(t)	37.0(t)	39.1(t)
42.3(t/s)	-	51.5(d)	52.8(q)
54.8(t)	56.3(q)	57.2(q)	-
60.4(d)	60.9(q)	61.3(t)	61.7(q)
67.0(d)	68.4(d)	68.5(d)	69.2(d)
69.7(d)	70.5(d)	71.1(d)	71.8(d)
72.1(s)	75.7(d)	75.8(d)	80.9(d)
82.8(s)	88.1(s)	93.5(s)	97.3(d)
99.6(d)	99.7(d)	100.8(s)	102.6(d)
-	123.4(d)	124.4(d)	126.2(d)
130.2(s)	131.0(s)	133.4(s)	139.1(s)
143.0(s)	145.1	150.6(s)	151.5(s)
154.5	192.0(s)	192.5(s)	

The LL-E33288 components are most conveniently separated and identified by high-performance liquid chromatography (HPLC) and by thin-layer chromatography (TLC). It is difficult, although not impossible, to separate the corresponding iodinated and brominated components by HPLC; however, they cannot be distinquished by TLC.

The preferred analytical separation of the LL-E33288-Br components by HPLC uses the following conditions:

Column:	"Separalyte C_{18} 5 m," 4.6 mm x 25 cm (Analytichem International);
Solvent:	Acetonitrile: 0.2M aqueous ammonium acetate (60:40);
Flow rate:	1.5 ml/minute
Detector:	Dual wavelength UV at 254 nm and 280 nm;
Sensitivity:	0-0.02 A.U.F.S.

Table IA gives the approximate retention times and volumes of LL-E33288β_1-Br, LL-E33288β_2-Br, and LL-E33288 γ_1-Br under these conditions.

TABLE IA

LL-E33288 Components	Retention Time (minutes)	Retention Volume(ml)
β_1-Br	5.7	8.6
β_2-Br	7.1	10.7
γ_1-Br	4.3	6.5

The preferred analytical HPLC separation of the iodine containing LL-E33288 components uses the following conditions:

Column:	NOVA-PAK C_{16} Radial-PAK cartridge with RCM-100 Radial Compression Module (Millipore, Waters Chromatography Division);
Solvent:	Acetonitrile: 0.2M aqueous ammonium acetate (50:50);
Flow Rate:	1.2 ml/minute;
Detector:	Dual wavelength UV at 254 nm and 280 nm;
Sensitivity:	0-0.02 A.U.F.S.

Table IB gives the approximate retention times and volumes of LL-E33288 α_1-I, LL-E33288α_2-I, LL-E33288α_3-I, LL-E33288 β_1-I, LL-E33288 β_2-I, LL-E33288 γ_1-I and LL-E33288δ_1-I under these conditions.

TABLE IB

LL-E33288 Components	Retention Time (minutes)	Retention Volume(ml)
α_1-I	11.9	14.3
α_2-I	9.1	10.9
α_3-I	1.5	1.8
β_1-I	4.4	5.3
β_2-I	5.0	6.0
γ_1-I	3.6	4.3
δ_1-I	2.6	3.1

The LL-E33288 components are separated and identified by the following TLC system:

Adsorbant: Silica gel 60 F_{254} pre-coated aluminum sheets, 0.2mm layer thickness, EM Reagents;

Detection: Visualized by quenching effect under short wavelength UV lamp (254 nm), and bioautography using <u>Bacillus subtilis</u> or the modified biochemical induction assay;

Solvent Systems: I, ethyl acetate saturated with 0.1<u>M</u> aqueous potassium dihydrogen phosphate;

II, 3% isopropyl alcohol in ethyl acetate saturated with 0.1<u>M</u> aqueous potassium dihydrogen phosphate;

III, ethyl acetate:methanol (95.5).

Table II gives the approximate Rf values of LL-E33288 components in these three systems:

<u>TABLE II</u>

LL-E33288 Components	R_f Value		
	Solvent System I	Solvent System II	Solvent System III
α_1-Br,α_1-I	0.67	0.80	0.79
α_2-Br,α_2-I	0.61	0.75	0.73
α_3-Br,α_3-I	0.55	0.69	0.61
α_4-Br	0.49	0.64	0.54
β_2-Br,β_2-I	0.32	0.41	0.45
β_1-Br,β_1-I	0.24	0.35	0.36
γ_1-Br,γ_1-I	0.18	0.28	0.27
δ_1-I	0.11	0.19	

The new antibacterial and antitumor agents designated LL-E33288α_1-Br, LL-E33288α_2-Br, LL-E33288α_3-Br, LL-E33288α_4-Br, LL-E33288 β_1-Br, LL-E33288β_2-Br, LL-E33288 γ_1-Br, LL-E33288α_1-I, LL-E33288α_2-I, LL-E33288 α_3-I, LL-EE288β_1-I, LL-E33288β_2-I, LL-E33288γ_1-I and LL-E33288δ_1-I are formed during the cultivation under controlled conditions of a new strain of <u>Micromonospora echinospora</u> ssp. <u>calichensis</u>. This microorganism is maintained in the culture collection of the Medical Research Division, American Cyanamid Company, Pearl River, New York as culture number LL-E33288. A viable culture of this new microorganism has been deposited with the Culture Collection Laboratory, Northern Regional Research Center, U. S. Department of Agriculture, Peoria, Illinois on August 9, 1984, and has been added to its permanent collection. It has been assigned by such depository the strain designation NRRL 15839. Access to such culture, under strain designation NRRL 15839, during pendency of the instant application shall be available to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 C.F.R. §1.14 and 35 U.S.C. §122, and all restrictions on availability to the public of such culture will be irrevocably removed upon grant of a patent on the instant application.

Culture LL-E33288 was isolated from a caliche clay soil sample collected in Texas.

The generic assignment of the strain NRRL 15839 to the genus Micromonospora was confirmed morphologically and chemically. The strain produces monospores either singly or in masses on the vegetative hyphae. No aerial hyphae were observed. Electron microscopic examination showed that the spores were warty. Whole cell analysis showed that the strain contained the meso isomer of diaminopimelic acid (DAP). The 3-OH derrivative of DAP was present in large (major) amounts. Additionally the strain showed the presence of xylose plus traces of arabinose in its whole cell sugar hydrolysates (whole cell sugar pattern of Type D).

From macromorphological and physiological studies it was concluded that NRRL 15839 can be considered subspecies of M. echinospora (it is closest to M. echinospora ssp. pallida). Data on the morphology of NRRL 15839 are given in Tables A and B. Physiological data are given in Tables C and D.

Table A

Macromorphology Of NRRL 15839
(Colors Are NBS-ISCC)

ISP Agar Medium	Spores	Vegetative Mycelium	Soluble Pigments
Yeast-Malt (ISP 2)	-	Dark orange-yellow (72)	-
Oatmeal (ISP 3)	-	Colorless → pale orange-yellow (73)	-
Inorganic Salts-Starch (ISP 4)	Slight border of black spores	Dark orange-yellow (72) to lt. yellow-brown (76)	Lt. brownish
Glycerol-Asparagine (ISP 5)	-	Pale orange-yellow (73) → colorless	-

Table B

Macromorphology of NRRL-15839 on Various Agar Media Used for Actinomycete Growth (28°C, 2 weeks)

Agar Medium	NRRL-15839
Pablum	Beige veg. Sl. black spores No sol. pig.
Yeast Czapeks	Beige veg. No spores No sol. pig.
Czapek's	Beige veg. Sl. black spores No sol. pig.
Yeast Dextrose	Tan veg. Moderate black sp. Sl. dark pig.
Nutrient	Colorless to tan veg. Sl. black spores No sol. pig.
Nutrient-Glycerol	Colorless to light beige veg. No black spores No sol. pig.
Bennett's Dextrin	Colorless to beige veg. Sl. black spores Sl. rosy-brown pig.
Glucose-Asparagine	Colorless to lt. orange-beige veg. No spores No sol. pig.

veg. = vegetative hyphae; pig. = pigment.

Table C

Carbohydrate Utilization of NRRL-15839

Arabinose	+
Cellulose	-
Fructose	+
Glucose	+
Inositol	-
Mannitol	-
Raffinose	±
Rhamnose	+
Sucrose	+
Xylose	+

Table D

Physiological Reactions of NRRL-15839

<u>Hydrolysis of</u>	
Casein	+
Xanthine	-
Hypoxanthine	-
Tyrosine	+
Adenine	-
Gelatin	+
Potato Starch	+
Esculin	+
<u>Production of</u>	
Nitrate Reductase	+
Phospatase	W
Urease	-

<u>Growth on</u>	
Salicin	-
5% NaCl	-
Lysozyme Broth	-
<u>Decarboxylation of</u>	
Acetate	+
Benzoate	-
Citrate	-
Lactate	-
Malate	-
Mucate	-
Oxalate	-
Propionate	+
Pyruvate	+
Succinate	-
Tartrate	-
<u>Acid from</u>	
Adonitol	-
Arabinose	+
Cellobiose	+
Dextrin	+
Dulcitol	-
Erythritol	-
Fructose	+
Galactose	V
Glucose	+
Glycerol	-
Inositol	-
Lactose	-
Maltose	+
Mannitol	-
Mannose	+
α-methyl D Glucoside	-
Melibiose	-
Raffinose	+
Rhamnose	+
Salicin	+
Sorbitol	-
Sucrose	+
Trehalose	+
Xylose	+
β-Methyl D-xyloside	-
<u>Growth at</u>	
10°	-
42°	+
45°	+

+ = positive; - = negative; V = variable;
W = weak.

Derivation of Mutant LL-E33288-R66, NRRL-15975

In an effort to improve fermentation yields, the orginal culture LL-E33288 (NRRL-15839) was plated and 50 single colonies were isolated. These were designated NS1 to NS50 (NS = natural selection).

Fermentation of these isolates showed that those with moderate sporulation were generally better producers of LL-E33288 complex. Selected as representative of this group was isolate NS6.

Using isolate NS6 as the starting culture, spore suspensions were prepared and expossed to various mutagens. Single colonies were isolated from a nitrosoguanidine treatment, but none proved to be significantly improved producers of the LL-E33288 complex. From a subsequent series of exposures to ultraviolet irradiation, single colonies were obtained from which isolate UV 610 was selected as a high yielding mutant. Isolate UV 610 was then streaked and sub-isolates 1 to 7 were obtained. Sub-isolate UV 610(3) was selected for further work.

Because of the highly potent antibacterial and antineoplastic nature of the LL-E33288 complex it is possible that once a limited concentration of the antibiotic is biosynthesized in the fermentation it may become toxic/inhibitory to the producing culture. Thus, an effort was made to obtain isolates which are resistant to the LL-E33288 antibiotic complex.

Vegetative growth from isolate UV 610(3) was prepared as employed for fermentation and used to inoculate a flask of medium consisting of peptone, dextrose, molasses and water. The medium was supplemented with LL-E33288β_1-Br at a concentration of 8 μg/m. A number of platings were done from this flask and a resistant population was obtained on the seventh day. A total of 97 colonies (R1 to R97) were isolated. Isolate R66 was selected as a potentially improved producer of LL-E33288 β_1-Br.

The history is represented schematically below.

```
E33288 (Wild type)
   |
   v
NS6 (Natural selection)
   |
   |--NTG [NG1-NG120](Nitrosoguanidine)
   |
   |--UV [UV1-UV200](Ultraviolet)
   |
   |--UV [UV201-UV400](Ultraviolet)
   |
   |--UV [UV401-UV702](Ultraviolet)
                 |
                 v
          UV610(3)(Sub-islate 3)
                 |
                 v
      [R1 to R97](Resistant colonies)
                 |
                 v
                R66
```

The mutant R66 is maintained in the culture collection of the Medical Research Division, American Cyanamid Company, Pearl river, New York as culture number LL-E33288 R66. A viable culture of this new microorganism has been deposited with the Culture Collection Laboratory, Northern Regional Research center, U. S. Department of Agriculture, Peoria, Illinois on June 6, 1985, and has been added to its permanent collection. It has been assigned by such depository the strain designation NRRL 15975. Access to such culture, under strain designation NRRL 15975, during pendency of the instant application

shall be available to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under C.F.R. §1.14 and 35 U.S.C. §122, and all restrictions on availability to the public of such culture will be irrevocably removed upon grant of a patent on the instant application.

Morphologically, NRRL-15975 forms fewer spores than NRRL-15839. A comparison of NRRL-15975 with NRRL-15839 is given in Table DD.

Chemically, both NRRL-15839 and NRRL-15975 show the same whole cell sugar patterns (Type D: xylose and traces of arabinose). The whole cell diaminopimelic acid analysis reveals that 15975 does not form the meso isomer but only the 3-hydroxy derivative (NRRL-15839 contains both compounds). This does not change the chemo-taxonomic assignment.

Physiological tests show that NRRL-15839 and NRRL-15975 differ in only two physiological reaction (See Table D). NRRL-15975 is negative for nitrate reductase and positive for utilization of lactate. NRRL-15839 was weakly positive for both, but is now negative after having been maintained on slants for a few months. Thus these characters should be considered variable for this taxon.

TABLE DD

Morphological Comparison of NRRL 15839 and NRRL 15975

Agar Medium		NRRL 15839	NRRL 15975
Bennett's-Dextrin	V[1]	Beige-tan	Beige tan
	Sp	Black, copious	None
	SS	None	None
Czapek's	V	Orange-tan	Orange-tan
	Sp	Black, traces	None
	SS	None	None
Yeast Extract-Czapek's	V	Orange tan, flat	Orange tan, convoluted
	Sp	Black, traces	None
	SS	None	Slight yellowish
Potato-Dextrose	V	Very poor growth	Very poor growth
	Sp	None	None
	SS	None	None
Nutrient glycerol	V	Tan	Tan
	Sp	Black, sparse	Black, sparse
	SS	None	Slight brownish
Nutrient	V	Tan	Tan
	Sp	Black, fair	None
	SS	None	None

1 = V = vegetative hyphae; Sp = spores; SS = soluble pigment.

It is to be understood that for the production of these new antibacterial and antitumor agents the present invention is not limited to this partucular organisms or to organisms fully answering the above growth microscropic characteristics which were given for illustrative purposes only. In fact, it is desired and intended to include the use of mutants produced from these organisms by various means such as exposure to X-radiation, ultraviolet radiation, N'-methyl-N'-nitro-N-nitrosoguanidine, actinophages and the like.

The *in vitro* antibacterial activity of LL-E33288 components was determined against a spectrum of gram-positive and gram-negative bacteria by a standard agar dilution method. Mueller-Hinton again containing two-fold decreasing concentrations of the antibiotics were poured into petri plates. the agar surfaces were inoculated with 1 to 5 x 10^4 colong forming units of bacteria by means of the steers replicating device. The lowest concentration of LL-E33288 component that inhibited growth of a bacterial strain after about 18 hours of incubation at approximately 35°C was recorded as the minimal inhibitory concentration (MIC) for that strain. The results are summarized in Table III.

TABLE III

In *vitro* Antibacterial Activity of LL-E33288 Components

Organism		Minimal Inhibitory Concentration, mcg/ml			
		β_1-Br	β_1-I	γ_1-Br	γ_1-I
Escherichia coli	CMC 84-11	0.25	0.50	0.50	0.25
" "	No. 311-(MP)	0.12	0.25	0.25	0.25
" "	ATCC 25922	0.12	0.25	0.25	0.25
Klebsiella pneumoniae	CMC 84-5	0.25	0.50	0.50	0.25
" "	AD (MP)	0.12	0.50	0.50	0.25
Enterobacter cloacae	CMC 84-4	0.5	0.50	0.50	0.50
" *aerogenes*	IO 83-44	0.25	0.25	0.50	0.50
Serratia marcescens	CMC 83-27	0.12	0.25	0.50	0.25
" "	F35 (MP)	0.12	0.50	0.25	0.12
Morganella morganii	10 83-18	0.5	1	0.50	0.25
Providencia stuartii	CMC 83-82	0.25	0.50	1	0.25
Citrobacter diversus	K 82-84	0.12	0.50	0.50	0.25
" *freundii*	IO 83-13	0.12	0.25	0.25	0.12
Acinetobacter sp.	CMC 83-89	0.06	0.25	0.25	0.12
" sp.	IO 83-49	0.12	0.25	0.25	0.06

TABLE III (continued

Organism		Minimal Inhibitory Concentration, mcg/ml			
		β1-Br	β1-I	γ1-Br	γ1-I
Pseudomonas aeruginosa	12-4-4 (MP)	0.5	0.50	1	0.25
" "	ATCC 27853	0.25	0.25	0.50	0.12
Staphylococcus aureus	Smith	≤0.0025	≤0.000031	≤0.000031	≤0.000031
" "	SSC 82-31	≤0.00025	≤0.000031	≤0.000031	≤0.000031
" "	ATCC 25923	≤0.00025	≤0.000031	≤0.000031	≤0.000031
" "	SSC 82-20	≤0.00025	≤0.000031	≤0.000031	≤0.000031
" "	SSC 82-26	≤0.00025	≤0.000031	≤0.000031	≤0.000031
" "	SSC 82-24	≤0.00025	≤0.000031	≤0.000031	≤0.000031
" "	SSC 82-57	≤0.00025	≤0.000031	≤0.000031	≤0.000031
Staphylococcus epidermidis	CMC-83-133	≤0.00025	≤0.000031	≤0.000031	≤0.000031
" "	ATCC 12228	≤0.00025	≤0.000031	≤0.000031	≤0.000031
Enterococcus sp.	CMC 83-53	0.0038	0.031	0.062	0.0078
Streptococcus faecalis	ATCC 29212	≤0.00025	0.00012	<0.000031	0.00012
Micrococcus luteus	PCI 1001	≤0.00025	≤0.000031	≤0.000031	≤0.000031
Bacillus subtilis	ATCC	≤0.00025	≤0.000031	≤0.000031	≤0.000031

Certain *in vivo* testing systems and protocols have been developed by the National Cancer Institute for testing compounds to determine their suitability as anti-neoplastic agents. These have been reported in "Cancer Chemotherapy Reports", Part III, Vol. 3, No. 2 (1972), Geran, *et al*. These protocols have established standardized screening tests which are generally followed in the field of testing for antitumor agents. Of these systems, lymphocytic leukemia

P388, melanotic melanoma B16, L1210 leukemia and colon 26 adenocarcinoma are particularly significant to the present invention. These neoplasms are utilized for testing as transplantable tumors in mice. Generally, significant anti-tumor activity, shown in these protocols by a percentage increase of mean survival times of the treated animals (T) over the control animals (C), is indicative of similar results in human leukemias and solid tumors.

Lymphocytic Leukemia P388 Test

The animals used were BDF_1 mice, all of one sex, weighing a minimum of 17 g and all within a 3 g weight range. There were 5 or 6 mice per test group. The tumor transplant was by intraperitoneal injection of 0.5 ml of dilute ascitic fluid containing 10^6 cells of lymphocytic leukemia P388. LL-E33288 antibiotics were tested in the P388 system both as the individual β_1-Br and $_1$-Br components and as a complex of all components (Bromo-complex). The test compounds were administered intrapertioneally at a volume of 0.5 ml in 0.2% Klucel in normal saline on days 1, 5 and 9 (relative to tumor inoculation) at the indicated doses. The mice were weighed and the survivors recorded on a regular basis for 30 days. The median survival time and the ratio of survival time for treated (T)/control (C) animals were calculated. The positive control compound was Cisplatin given as an intraperitioneal injection in 0.5 ml of 0.2% Klucel on days 1, 5 and 9 at the indicated doses. The results appear in Table IV.

If T/C X 100 (%) is 125 or over, the tested compound is considered to have significant anti-tumor activity.

TABLE IV

Lymphocytic Leukemia P388 Test

Compound	Dose (mg/kg)	Median Survival (Days)	T/C x 100 (%)
LL-E33288 (Bromo-complex)	3.2	16.5	156
	1.6	17.5	165
	0.8	18.5	175
	0.4	19	179
	0.2	16.5	156
Control	-	10.6	-
Positive Control	1.0	21.5	203
	0.25	15	142
	0.06	14.5	137
LL-E-33288β_1-Br	0.4	13	105
	0.2	18	145
	0.1	19	153
	0.05	17.5	141
	0.025	18	145
	0.012	14	113
Control	-	12.4	-
Positive Control	1.0	25.5	206
	0.4	19	153
	0.06	15	121

TABLE IV (continued)

Compound	Dose (mg/kg)	Median Survival (Days)	T/C x 100 (%)
LL-E33288γ_1-Br	0.2 0.1 0.05 0.025 0.012	14 21 19.5 18 14.5	113 169 157 145 117
Control	-	12.4	-
Positive Control	1.0 0.4 0.06	25.5 19 15	206 153 121

Melanotic Melanoma B16

The animals used were BDF_1 mice, all of the same sex, weighing a minimum of 17 g and all within a 3 g weight range. There are normally 6 animals per test group. A 1 g portion of melanotic melanoma B_{16} tumor was homogenized in 10 ml of cold balanced salt solution and a 0.5 ml aliquot of the homogenate was implanted intraperitoneally into each of the test mice. LL-E33288 antibiotics were tested in the B_{16} system both as the individual β_1-Br and γ_1-Br components and as a complex of all components (Bromo-complex). The test compounds were administered intraperitioneally on days 1 through 9 (relative to tumor inoculation) at various doses. The mice were weighed and survivors recorded on a regular basis for 60 days. The median survival time and the ratio of survival time for treated (T)/control (C) animals were calculated. The positive control compounds were Cisplatin or Adriamycin. The results of this test appear in Table V. If T/C X 100 (%) is 125 or over, the tested compound is considered to have significant anti-tumor activity.

TABLE V

Melanotic Melanoma B_{16} Test

Compound	Dose (mg/kg)	Median Survival (Days)	T/C x 100 (%)
LL-E33288 (Bromo-complex)	0.8 0.4 0.2 0.1	30 29.5 27 24	188 184 169 150
Control	-	16	-
Cisplatin	0.4 0.2 0.1 0.05	25 25 23 21.5	156 156 144 134
LL-E33288β_1-Br	0.05 0.025 0.0125	32 33.5 32	168 176 168
Control	-	19	-
Adriamycin	0.8 0.4	> 60 > 60	> 316 > 316

TABLE V (continued)

Compound	Dose (mg/kg)	Median Survival (Days)	T/C x 100 (%)
LL-E33288γ_1-Br	0.05 0.025 0.0125	33.5 30 37	176 159 195
Control	-	19	-
Adriamycin	0.8 0.4	> 60 > 60	> 316 > 316

Lymphocytic Leukemia L1210 Test

The animals used were BDF$_1$ mice, all of one sex, weighing a minimum of 17 g and all within a 3 g weight range. There were 6 mice in each test group and 18 in control groups. The tumor transplant was by intraperitoneal injection of 0.5 ml of lymphocytic leukemia L1210 at a concentration of 10^5 cells per mouse. LL-E33288 antibiotics were tested in the L1210 system both as the individual β_1-Br component and as a complex of all components (Bromo-complex). The test compounds were administered on days 1, 5 and 9 or days 1 through 9 (relative to tumor inoculation) at various doses. The mice were weighed and survivors recorded on a regular basis for 30 days. The median survival time and the ratio of survival time for treated (T)/control (C) animals were calculated. The positive control compound was 1,4-dihydroxy-5,8-bis[[2-(2-hy-

droxyethylamino)ethyl]amino]anthraquinone dihydrochloride or Cisplatin given intraperitoneally at the indicated dose. The results appear in Table VI. If T/C X 100 (%) is 125 or over, the tested compound is considered to have significant anti-tumor activity.

TABLE VI

Lymphocytic Leukemia L1210 Test

Compound	Dose (mg/kg)	Median Survival (Days)	T/C x 100 (%)
LL-E33288 (Bromo-complex)	1.5 0.8 0.4 0.2	29 28.5 22.5 22.5	174 171 135 135
Control	-	16.7	-
Anthraquinone	1.6 0.8 0.4	30 30 30	180 180 180
LL-E33288β_1-Br	0.2 0.1 0.05 0.025	11.3 11.4 11 11.3	136 137 133 136
Control	-	8.3	-
Cisplatin	5 2.5 1.25	7.5 12 11	90 145 133

Colon 26 Adenocarcinoma Test

The animals used were CD_2F_1 female mice weighing a minimum of 17 g and all within a 3 g weight range. There were 5 or 6 mice per test group with three groups of 5 or 6 animals used as untreated controls for each test. The tumor implant was by intraperitoneal (or subcutaneous) injection of 0.5 ml of a 2% Colon 26 tumor brei in Eagle's MEM medium containing antibiotics. LL-E33288 antibiotics were tested in the Colon 26 system as a complex (Bromo-complex) of all components. The test compounds were administered intraperitoneally on days 1, 5 and 9 (relative to tumor implant doses). The mice were weighed and deaths recorded on a regular basis for 30 days. The median survival times for treated (T)/control (C) animals were calculated. The positive control compound was Cisplatin. The results appear in Table VII. If T/C X 100 (%) is 130 or over, the tested compound is considered to have significant antitumor activity.

TABLE VII

Colon 26 Adenocarcinoma Test

Compound	Dose (mg/kg)	Median Survival (Days)	T/C x 100 (%)
LL-E33288 (Bromo-complex)	1.5	39.5	212
	0.8	32.5	175
	0.4	34	183
	0.2	25.5	137
Control	-	18.6	-
Positive Control	1	29	156
	0.5	38.5	207
	0.25	37.5	202

M5076 Sarcoma

The M5076 reticular cell Sarcoma is propagated as subcutaneous implants in C57B2/6 female mice. In the assays for antitumor activity, BDF_1 mice of either sex were inoculated intraperitoneally with 0.5 ml of a 10% tumor brei. LL-E33288 antibiotics were tested in the M5076 system as a complex (Bromo-complex) of all components. Test compounds were administered intraperitoneally on days 1, 5, 9, 13 and 17 relative to tumor inoculation on day zero. The median survival time in days was determined for each drug dose used on day 60 and the ratio of survival time for treated (T)/control (C) animals were calculated.

The results of this test appear in Table VIII compared to the results obtained with Cisplatin. If T/C X 100 (%) is 125 or over, the tested compound is considered to have significant anti-tumor activity.

TABLE VIII

M5076 Sarcoma

Compound	Dose (mg/kg)	Median Survival (Days)	T/C x 100 (%)
LL-E33288 (Bromo-complex)	1.5	50	175
	0.8	50	175
	0.4	39.5	139
Control	-	28.5	-
Cisplatin	1	30	105
	0.5	44.5	156
	0.25	45	158

In the same manner, the following iodo-components were tested for antineoplastic activity.

TABLE IX

Lymphocytic Leukemia P388 Test

Compound	Dose (mg/kg)	Median Survival (Days)	T/C x 100 (%)
LL-E33288 γ_1-I (Test 1)	.005	>25.5	>196
	.0025	22	169
	.00125	18.5	142
	.0006	18	138
	.0003	15.5	119
	.00015	15	115
Control	-	13	-
Positive Control Novantrone® *	1.6	22.5	173
LL-E33288 γ_1-I (Test 2)	.01	11	100
	.005	18	164
	.0025	22.5	205
	.00125	18.5	168
	.0006	16	145
	.0003	14	127
	.00015	14	127
Control	-	11	-
Positive Control Novantrone®	1.6	19	173
	0.8	16	145

*1,4-dihydroxy-5,8-bis[[2-(2-hydroxyethylamino)ethyl]amino]anthraquinone-2HCl

TABLE X

Melanotic Melanoma B_{16} Test

Compound	Dose (mg/kg)	Median Survival (Days)	T/C x 100 (%)
LL-E33288 γ_1-I	.0025 .00125 .0006 .0003 .00015 .00007	26.5 27 42.5 35.5 33.5 30.5	156 159 250 209 197 179
Control	-		-
Adriamycin	0.8 0.4 0.2	48 40 34.5	282 235 203

TABLE XI

Lymphocytic Leukemia L1210 Test

Compound	Dose (mg/kg)	Median Survival (Days)	T/C x 100 (%)
LL-E33288 γ_1-I	.01	8	89
	.005	14	156
	.0025	11	122
	.0012	10.5	117
	.0006	10	111
Control	-	9	-
Positive Control Novantrone®	3.2	15	167
	1.6	11.5	128
	0.8	12	133
	0.4	11	122

TABLE XII

Colon 26 Adenocarcinoma Test

Compound	Dose (mg/kg)	Median Survival (Days)	T/C x 100 (%)
LL-E33288 γ_1-I	.01	11	59
	.005	25.5	138
	.0025	27	146
	.00125	22.5	122
	,0006	23.5	127
	.0003	20	108
	.00015	17.5	95
	.00007	17	92
Control	-	18.5	-
Positive Control Cisplatin	2	15.5	84
	1	27.5	149
	0.5	23.5	127

General Fermentation Conditions

Cultivation of Micromonospora echinospora NRRL 15839 or NRRL 15975 may be carried out in a wide variety of liquid culture media. Media which are useful for the production of these novel antibacterial and antitumor agents include an assimilable source of carbon, such as starch, sugar, molasses, glycerol, etc.; an assimilable source of nitrogen such as protein, protein hydrolysate, polypeptides, amino acids, corn steep liquor, etc.; and inorganic anions and cations, such as potassium, sodium, ammonium, calcium, sulfate, carbonate, phosphate, chloride, etc. and sources of either bromine (sodium bromide) or iodine (potassium iodide). Trace elements such as boron, molybdenum, copper, etc., are supplied as impurities of other constituents of the media. Aeration in tanks and bottles is supplied by forcing sterile air through or onto the surface of the fermenting medium. Further agitation in tanks is provided by a mechanical impeller. An antifoam agent such as silicone may be added as needed.

General Procedure for the Isolation and Separation of the Antibiotics - LL-E33288

The LL-E33288 antibiotics are recovered from the fermentation broth by extracting the whole mash with an organic solvent such as ethyl acetate or dichloromethane. The antibiotic complex, contained in the organic extract, is further purified by selective precipitation from lower hydrocarbons. The crude LL-E-33288 antibiotic complex thus obtained is further purified and separated into the individual components by a series of column chromatographies using silica gel, Sephadex® LH-20 (Pharmacia Fine Chemicals) and C_{18} bonded silica.

The invention will be described in greater detail in conjunction with the following non-limiting specific examples.

Example 1

Inoculum Preparation

A typical medium used to grow the primary inoculum was prepared according to the following formula:

Beef extract................	about 0.3%
Tryptone....................	about 0.5%
Dextrose....................	about 0.5%
Dextrin.....................	about 2.4%
Calcium carbonate...........	about 0.4%
Yeast extract...............	about 0.5%
Water.......qs to...........	100%

This medium was adjusted to pH 7.0 and then sterilized. A 100 ml portion of this sterile medium, in a flask, was inoculated with frozen mycelia of the culture NRRL 15839. The inoculated medium was placed on a rotary shaker and agitated vigorously for 48 hours at 32°C. This incubated medium was then used to inoculate 10 liters of the above sterile medium in a 14 liter fermentor. This medium was incubated, with agitation, at 32°C for 48 hours, providing secondary inoculum. This secondary inoculum was then used to inoculate 300 liters of the above sterile medium in a tank and incubated for 48 hours at 30°C while agitated by an impeller driven at 180-200 rpm, providing the tertiary or seed inoculum.

Example 2

Tank Fermentation

A fermentation medium was prepared according to the following formulation:

Dextrose..................................	about 0.5%
Sucrose...................................	about 1.5%
Peptone, bacteriological grade..........	about 0.2%
Dibasic potassium phosphate.............	about 0.01%

Molasses	about 0.5%
Calcium carbonate	about 0.5%
Source of bromine or iodine	trace amounts
Water qs to	100%

A 2800 liter portion of the above medium was sterilized and then inoculated with 300 liters of tertiary (seed) inoculum prepared as described in Example 1. Aeration was supplied at the rate of 0.53 liters of sterile air per liter of mash per minute and agitation was supplied by an impeller driven at 110 rpm. The temperature was maintained at about 28°C and the fermentation was terminated after about 97 hours, at which time the mash was harvested.

The fermentation was monitored for production of the LL-E33288 antibiotics by antibacterial activity, biochemical induction assay, TLC and HPLC analyses.

The whole harvest mash was adjusted to pH 6 and then extracted with 1/2 mash volume ethyl acetate. The ethyl acetate extract was concentrated to a syrup which was washed twice with hexane and filtered through diatomaceous earth. The diatomaceous earth cake was thoroughly mixed with ethyl acetate and filtered. The filtrate was concentrated to 3 liters, dried over excess anhydrous sodium sulfate and then precipitated by the addition of hexane giving about 26.7 g of crude LL-E33288 complex.

Example 3

Separation of LL-E33288 α_1-Br, α_2-Br, α_3-Br and α_4-Br from LL-E33288 β_1-Br, β_2-Br and γ_1-Br

The approximately 26.7 g of crude LL-E33288 complex (Bromo-complex) from Example 2 was divided evenly into three portions and chromatographed on three separate 2.4 x 110 cm silica gel columns (Silica Woelm®, 32-63 m, Woelm Pharma) packed and equilibrated with ethyl acetate saturated with 0.1$\underline{M}$ aqueous potassium dihydrogen phosphate. The columns were first eluted with the same solvent at a flow rate of 3 ml/minute

for 18 hours, collecting 18 ml fractions. The eluent was changed to ethyl acetate:methanol (95:5) and elution continued for 8 hours. Finally the columns were eluted with ethyl acetate:methanol (90:10) for 10 hours. The fractions were assayed by the modified biochemical induction assay (BIA). The positive fractions were analysed by TLC using silica gel 60 precoated sheets and developed with the solvent system 3% isopropanol in ethyl acetate saturated with 0.1M aqueous potassium dihydrogen phosphate and detected by bioautography using the modified BIA.

Fractions containing LL-E33288α_1-Br,α_2-Br, α_3-Br and α_4-Br (LL-E33288α-Br complex) from the three columns were pooled, concentrated to dryness and the residue was dissolved in ethyl acetate and washed with a small amount of water. The ethyl acetate solution was dried over anhydrous sodium sulfate and precipitated as before to yield about 4.2 g of crude LL-E33288α-Br complex.

Fractions containing LL-E33288β_2-Br, β_1-Br and γ_1-Br (LL-E33288 β-Br complex containing γ-Br from the three columns were pooled and worked up as above to yield about 2.0 g of crude LL-E33288 β-Br complex containing γ-Br.

Example 4

Isolation of LL-E33288 β_1-Br and LL-E33288 γ_1-Br

An approximately 1.9 g sample of the LL-E33288β-Br complex containing γ-Br from Example 3 was chromatographed on a 25 x 10 cm Sephadex® LH-20 column equilibrated with methanol:water (90:10) at a flow rate of 1.2 ml/minute, collecting 15 ml fractions. The fractions were assayed in the BIA and those active were analysed by TLC as before. Fractions 21-26 containing most of the LL-E33288β_1-Br, β_2-Br and γ_1-Br, were pooled and concentrated to remove methanol and the resulting aqueous mixture was lyophilized to yield about 435 mg of partially purified complex containing approximately 10% of LL-E33288 β_1-Br, 1% of LL-E33288β_2-Br and 4% of LL-E33288 γ_1-Br.

The above partially purified LL-E33288β-Br complex containing γ-Br was divided evenly and chromatographed on two 1.5 x 100 cm silica gel columns (Kiesel Gel 60, 40-63 μm, EM Products for chromatography) packed and equilibrated with ethyl acetate:methanol (98.2) at a flow rate of 1 ml/minute, collecting 12 ml fractions. The fractions were assayed and analysed by TLC as before and those containing primarily LL-E33288β_1-Br were pooled, concentrated and precipitated from hexane to yield about 26 mg of 80% pure LL-E33288β_1-Br. Those fractions containing LL-E33288γ_1-Br (chromatographing just after LL-E33288 β_1-Br) were pooled and worked up to yield about 4.5 mg of 30% pure LL-E33288γ_1-Br. A few fractions containing LL-E33288β_2-Br (chromatographing just before LL-E33288-β_1-Br), were pooled and worked up to yield a trace amount of LL-E33288β_2-Br.

Example 5

Final Purification of LL-E33288β_1-Br

The approximately 26 mg of 80% pure LL-E33288β_1-Br from Example 4 was combined with other LL-E33288 β_1-Br samples of similar purity derived from other fermentations conducted under identical conditions. A total of about 38 mg of this combined β_1-Br was further purified by reverse phase preparative TLC using Whatman PLKC$_{18}$F, 100 m precoated TLC plates, developed with methanol:0.1M ammonium acetate buffer at pH 4.0 (90:10). The band containing LL-E33288 β_1-Br, chromatographing at R_f=0.66 and visualized by quenching effect under short wavelength UV lamp (254 nm), was excised and the antibiotic was washed off the adsorbant with 10% isopropyl alcohol in ethyl acetate saturated with 0.1M aqueous potassium dihydrogen phosphate. The solution was concentrated and the residue was dissolved in ethyl acetate and washed with a small amount of water. The organic solution containing LL-E33288 β_1-Br was worked up as before to yield about 24.5 mg of 90% pure LL-E33288 β_1-Br. This sample was further purified by preparative TLC on silica gel (Silica

Gel GF precoated plates, 1000 m, Analtech) developed with 3% isopropyl alcohol in ethyl acetate saturated with 0.1M aqueous potassium dihydrogen phosphate. The major quenching band under short wavelength UV lamp (254 nm), chromatographing at R_f=0.7, was excised and the antibiotic was washed off the adsorbant with dichloromethane:methanol (80:20). The organic solution containing LL-E33288 β_1-Br was worked up as before to yield about 18.8 mg of substantially pure LL-E33288 β_1-Br.

Example 6

Final Purification of LL-E33288 β_1- Br

The approximately 4.5 mg of 30% pure LL-E33288 γ_1-Br from Example 4 was combined with other LL-E33288 γ_1-Br samples of similar purity derived from other fermentations conducted under identical conditions. A total of 18 mg of this combined sample was further purified by preparative TLC on silica gel (Silica Gel GF precoated tapered plates, Analtech) developed with 2% isopropyl alcohol in ethyl acetate saturated with 0.1M aqueous potassium dihydrogen phosphate. The major quenching band under short wavelength UV lamp (254 nm), chromatographing at R_f=0.5, was excised and worked up as before to yield about 4.3 mg of substantially pure LL-E33288 γ_1-Br.

A preferred fermentation medium for production of LL-E33288 Bromo-complex is as follows:

Ingredient	Percent
Sucrose	2.0
Ferrous Sulfate Heptahydrate	0.01
Magnesium Sulfate Heptahydrate	0.02
Calcium Carbonate	0.5
Peptone	0.2
Molasses	0.5
Sodium Bromide	0.05
Water qs to	100

However, the addition of iodine as potassium iodide, to the fermentation medium provided substantial improvements by:

1) markedly enhancing vegetative growth in the fermentation;

2) increasing zones of inhibition in bioassays versus <u>Escherichia coli</u> #300 and <u>Bacillus subtilis</u> #308;

3) providing substantial activity at the Rf of LL-E33288β_1-I and γ_1-I on the bioautography of TLC plates; and

4) enhancement of other components as detected by TLC.

The following two media are preferred for the production of LL-E33288 Iodo-complex:

Ingredient	Percent Media A	Percent Media B
Sucrose	2.0	2.0
Ferrous Sulfate Heptahydrate	0.01	0.01
Magnesium Sulfate Heptahydrate	0.02	0.02
Calcium Carbonate*	0.5	0.25
Peptone**	0.2	0.2
Molasses	0.5	0.5
Potassium iodide	0.05	0.01
Water qs to	100	100

* Mississippi lime.

** Best results were obtained with MARCOR® bacteriological peptone, but other peptones usable and also polypeptides from meat and casein hydrolyzates.

Example 7

A mycelial-spore suspension was prepared by scraping the surface of a slant of culture NRRL-15839 to which 5 ml of sterile distilled water had been added. This suspension was then used to inoculate 100 ml of sterile seed medium of the following formula:

Yeast Extract	0.5%
Beef Extract	0.3%
Tryptose	0.5%
Starch	2.4%
Dextrose	0.5%
Calcium Carbonate	0.4%
Water qs to	100.0%

in a 500 ml flask. This seed flask was incubated at 28°C on a rotary shaker at 200 rpm for 3-4 days, producing Stage I inoculum.

The Stage I inoculum was used to inoculate a Stage II inoculum of the same sterile medium, which was incubated under the same conditions for 2 days.

The Stage II inoculum was then used to inoculate 100 ml of sterile fermentation medium of the formula:

Sucrose	2.0%
Ferrous Sulfate Heptahydrate	0.01%
Magnesium Sulfate Heptahydrate	0.02%
Calcium Carbonate	0.5%
Peptone (MARCOR®)	0.2%
Molasses	0.5%
Potassium Iodide	0.05%
Water qs to	100.0%

This medium was incubated at 28°C on a shaker at 200 rpm for 5 days at which time the mash was harvested.

A concentration of 4 to 20 μg/ml of potassium iodide appears to be optimal, but concentrations of 2 mg/ml do not appear to depress yields.

NRRL-15839 can be induced to produce LL-E33288 β_1-I when potassium iodide is present in the medium, but only at very low levels (0.2-0.3 μg/ml) as against 1.5-3.5 μg/ml for the better producing NRRL-15975.

Yields of β_1-I and γ_1-I in an iodine medium are 2 to 8 times greater than yields of corresponding brominated compounds β_1-Br and γ_1-Br in a bromine medium using NRRL-15975.

Example 8

Separation of LL-E33288 α_1-I, α_2-I, and α_3-I from LL-E33288 β_1-I, β_2-I, γ_1-I and δ_1-I

Approximately 41.3 g of crude LL-E33288 complex derived from the processing 7500 liters of a fermentation using NRRL-15975 and medium containing inorganic iodide was divided evenly into two portions and chromatographed on two separate 2.5 x 110 cm silica gel column (Silica Woelm, 32-63 um) packed and equilibrated with ethyl acetate. The columns were first eluted with ethyl acetate at a flow rate of 4 ml/minute for 4 hours, collecting 20 ml fractions. The eluent was changed to a concave gradient from ethyl acetate saturated with 0.1 M aqueous potassium dihydrogen phosphate to 10% isopropyl alcohol in ethyl acetate saturated with 0.1 M aqueous potassium dihydrogen phosphate over 24 hours. The columns were finally eluted with 10% isopropyl alcohol in ethyl acetate saturated with 0.1 M aqueous potassium dihydrogen phosphate over night. The fractions were assayed in the BIA and those active were analysed by TLC as described in Example 3.

Fractions (86-107) containing LL-E33288α_3-I from the two columns were pooled and worked up as before to yield about 2.1 g of crude LL-E33288α_3-I.

Fractions (182-253) containing LL-E33288α_1-I and α_2-I from the two columns were pooled and worked up to yield about 4.2 g of a crude mixture of LL-E33288 α_1-I and α_2-I.

Fractions (254-272) containing LL-E33288β_2-I and β_1-I from the two columns were pooled and worked up to yield about 1.2 g of a crude mixture of LL-E33288 β_2-I and β_1-I.

Fractions (273-302) containing LL-E33288 γ_1-I from the two columns were pooled and worked up to yield about 1.9 g of 30% pure LL-E33288 γ_1-I.

Fractions (303-340) containing LL-E33288δ_1-I from the two columns were pooled and worked up to yield about 1.3 g of partially purified LL-E33288 δ_1-I.

Example 9

Purification of LL-E33288 γ_1-I

Approximately 900 mg of the 30% pure LL-E33288 γ_1-I from Example 8 was chromatographed on a 2.5 x 120 cm sephadex LH-20 column equilibrated with ethyl acetate:dichloromethane:ethanol (2:2:1) at a flow rate of 1 ml/minute, collecting 12 ml fractions. The fractions were assayed and analysed by TLC as before and those containing LL-E33288γ_1-I (fractions 24-33) were pooled and worked up to yield 428 mg of 64% pure LL-E33288γ_1-I.

A 22 mg sample of the above was chromatographed on a 0.8 x 24 cm Sepralyte C_{18} (35-60 um, Analytichem) column equilibrated with acetonitrile:0.2 M aqueous ammonium acetate (55.45) at a flow rate of 2 ml/minute, collecting 12 ml fractions. The fractions were assayed and analysed by TLC as before and those containing pure LL-E33288γ_1-I were pooled and worked up to yield 7.7 mg of pure LL-E33288 γ_1-I.

Example 10

Purification of LL-E33288β_1-I and β_2-I

Approximately 600 mg of the crude mixture of LL-E33288 β_2-I and β_1-I from Example 8 was chromatographed on a 2.5 x 120 cm Sephadex LH-20 column equilibrated with ethyl acetate:dichloromethane:ethanol (2:2:1) at a flow rate of 1 ml/minute, collecting 12 ml fractions. The fractions were assayed and analysed by TLC as before and those containing LL-E33288 β_2-I and LL-E33288 β_1-I (fractions 23-31) were pooled and worked up to yield 81 mg of a partially purified mixture of LL-E33288 β_2-I and β_1-I.

The sample above was chromatographed on a 1.5 x 90 cm Sephadex LH-20 column equilibrated with hexane: dichloromethane:ethanol (3:1:1) at a flow rate of 0.8 ml/minute, collecting 12 ml fractions. The fractions were assayed and analysed by TLC as before and those containing LL-E33288 β_2-I (fractions 17-30) and LL-E33288 β_1-I (fractions 31-38) were pooled separately and worked up to yield 31 mg of partially purified LL-E33288 β_2-I and 20 mg of 80% pure LL-E33288 β_1-I.

[redacted] CLAIMS

[redacted]

1. A compound LL-E33288α_1-Br, having the following R_f values in the indicated solvent systems on TLC on silica gel sheets:

a) ethyl acetate saturated with 0.1<u>M</u> aqueous potassium dihydrogen phosphate, R_f=0.67;

b) 3% isopropanol in ethyl acetate saturated with 0.1<u>M</u> aqueous potassium dihydrogen phosphate, R_f=0.80; and

c) ethyl acetate:methanol (95:5), R_f=0.79.

2. A compound LL-E33288α_2-Br, having the following R_f values in the indicated solvent systems on TLC on silica gel sheets:

a) ethyl acetate saturated with 0.1<u>M</u> aqueous potassium dihydrogen phosphate, R_f=0.61;

b) 3% isopropanol in ethyl acetate saturated with 0.1<u>M</u> aqueous potassium dihydrogen phosphate, R_f=0.75; and

c) ethyl acetate:methanol (95:5), R_f=0.73.

3. A compound LL-E33288α3-Br having the following R_f values in the indicated solvent systems on TLC on silica gel sheets:

a) ethyl acetate saturated with 0.1M aqueous potassium dihydrogen phosphate, R_f=0.55;

b) 3% isopropanol in ethyl acetate saturated with 0.1M aqueous potassium dihydrogen phosphate, R_f=0.69; and

c) ethyl acetate:methanol (95:5), R_f=0.61.

4. A compound LL-E33288α_4-Br, having the following R_f values in the indicated solvent systems on TLC on silica gel sheets:

a) ethyl acetate saturated with 0.1M aqueous potassium dihydrogen phosphate, R_f=0.49;

b) 3% isopropanol in ethyl acetate saturated with 0.1M aqueous potassium dihydrogen phosphate, R_f=0.64; and

c) ethyl acetate:methanol (95:5), R_f=0.54.

5. A compound LL-E33288β_1-Br,

a) having an approximate elemental analysis: C 48.6; H 5.6; N 2.9; S 9.1 and Br 5.5;

b) having a melting point: 146-150°C (dec.);

c) having a specific rotation: $[\alpha]_D^{26} = -49\pm10°$ (0.1%, ethanol);

d) having ultraviolet absorption spectra as shown in Figure I of the drawings;

e) having an infrared absorption spectrum as shown in Figure II of the drawings;

f) having a proton magnetic resonance spectrum as shown in Figure III of the drawings;

g) having a carbon-13 magnetic resonance spectrum as shown in Figure IV of the drawings with significant peaks at:

17.60(q);	17.64(q);	18.9(q);	19.7(q);
22.4(q);	22.8(q);	23.5(q);	34.3(t);
36.9(t);	39.2(t/d);	47.8(d);	51.7(q);
52.7(q);	54.6(d)	56.3(q);	57.2(q);
57.8(d);	61.0 (q/d);	61.7(d);	62.4(t);
66.9(d);	68.4(d);	69.1(d);	69.7(d);
70.2(d);	71.1(d);	71.9(d);	72.1(s);
76.1(d);	81.0(d);	83.3(s);	88.2(s);
97.4(d);	99.7(d);	100.8(s);	102.5(d);
115.1(s);	123.4(d);	124.4(d);	126.5(d);
130.2(s);	130.8(s);	144.6(s);	149.3(s);
149.5(s);	191.7(s);	192.4(s);	and

h) having the following R_f values in the indicated solvent systems on TLC on silica gel sheets:

i) ethyl acetate saturated with 0.1<u>M</u> aqueous potassium dihydrogen phosphate, R_f=0.24;

ii) 3% isopropanol in ethyl acetate saturated with 0.1<u>M</u> aqueous potassium dihydrogen phosphate, R_f=0.35;

iii) ethyl acetate:methanol (95:5), R_f=0.36.

i) having a molecular weight: 1333/1335, respectively for $^{79}Br/^{81}Br$; and

j) having a molecular formula: $C_{54}H_{84}N_3O_{22}S_4Br$.

6. A compound LL-E33288β_2-Br, having the following R_f values in the indicated solvent systems on TLC on silica gel sheets:

a) ethyl acetate saturated with 0.1M aqueous potassium dihydrogen phosphate, R_f=0.32;

b) 3% isopropanol in ethyl acetate saturated with 0.1M aqueous potassium dihydrogen phosphate, R_f=0.41; and

c) ethyl acetate:methanol (95:5), R_f=0.45.

7. A compound LL-E33288γ_1-Br

a) having ultraviolet absorption spectra as shown in Figure V of the drawings;

b) having an infrared absorption spectrum as shown in Figure VI of the drawings;

c) having a proton magnetic resonance spectrum as shown in Figure VII of the drawings; and

d) having the following R_f values in the indicated solvent systems on TLC on silica gel sheets:

i) ethyl acetate saturated with 0.1M aqueous potassium dihydrogen phosphate, R_f=0.18;

ii) 3% isopropanol in ethyl acetate saturated with 0.1M aqueous potassium dihydrogen phosphate, R_f=0.28;

iii) ethyl acetate:methanol (95:5), R_f=0.27.

e) having a carbon-13 magnetic resonance spectrum as shown in Figure VIII of the drawings with significant peaks at:

14.4	17.6	17.9	19.0
19.7	-	22.8	-
-	34.0	37.6	39.5
42.1	-	51.6	52.7
54.1	56.3	57.3	-
59.3	61.1	61.8	61.9
67.2	68.18	68.23	69.7
70.1	70.8	71.1	71.7
71.8	76.1	-	81.0
82.9	88.4	-	97.8
100.0	100.2	101.3	103.0
115.3	123.0	124.9	126.9
130.4	131.1	131.8	138.0
144.7	-	149.5	149.6
155.6	192.5	192.9	

f) having a molecular formula: $C_{53}H_{82}N_3O_{22}S_4Br$; and

g) having a molecular weight: 1319/1321, respectively for $^{79}Br/^{81}Br$.

8. A compound LL-E33288 α_1-I

a) having the following R_f values in the indicated solvent systems on TLC on silica gel sheets:

i) ethyl acetate saturated with 0.1M aqueous potassium dihydrogen phosphate, R_f=0.67;

ii) 3% isopropanol in ethyl acetate saturated with 0.1$\underline{M}$ aqueous potassium dihydrogen phosphate, R_f=0.80; and

iii) ethyl acetate:methanol (95:5), R_f=0.80; and

b) having a molecular weight:1145.

9. A compound LL-E33288α_2-I
a) having the following R_f values in the indicated solvent systems on TLC on silica gel sheets:

i) ethyl acetate saturated with 0.1$\underline{M}$ aqueous potassium dihydrogen phosphate, R_f=0.61;

ii) 3% isopropanol in ethyl acetate saturated with 0.1$\underline{M}$ aqueous potassium dihydrogen phosphate, R_f=0.75; and

iii) ethyl acetate:methanol (95:5), R_f=0.73;

b) containing only the following elements: C, H, N, O, S and I;

c) having a molecular weight:1131; and

d) having a proton magnetic resonance spectrum as shown in Figure IX of the drawings.

10. A compound LL-E33288α_3-I
a) having the following R_f values in the indicated solvent systems on TLC on silica gel sheets:

i) ethyl acetate saturated with 0.1$\underline{M}$ aqueous potassium dihydrogen phosphate, R_f=0.55;

ii) 3% isopropanol in ethyl acetate saturated with 0.1<u>M</u> aqueous potassium dihydrogen phosphate, R_f=0.69; and

iii) ethyl acetate:methanol (95:5), R_f=0.61;

b) having a molecular weight:1066; and

c) having a proton magnetic resonance spectrum as shown in Figure X of the drawings.

11. A compound LL-E33288β_1-I

a) having the following R_f values in the indicated solvent systems on TLC on silica gel sheets:

i) ethyl acetate saturated with 0.1M aqueous potassium dihydrogen phosphate, R_f=0.24;

ii) 3% isopropanol in ethyl acetate saturated with 0.1M aqueous potassium dihydrogen phosphate, R_f=0.35; and

iii) ethyl acetate:methanol (95:5), R_f=0.36;

b) having an ultraviolet absorption spectra as shown in Figure XI of the drawings;

c) having an infrared absorption spectrum as shown in Figure XII of the drawings;

d) having a proton magnetic resonance spectrum as shown in Figure XIII of the drawings;

e) having a carbon 13 magnetic resonance spectrum as shown in Figure XIV of the drawings with significant peaks at

-	17.5	17.6	18.9
-	22.4	22.8	23.4
25.4	34.3	36.9	39.2
-	47.9	51.6	52.8
54.8	56.3	57.2	57.9
60.9		61.6	62.2
67.0	68.4	68.4	69.1
69.6	70.4	71.1	71.8
72.2	76.2	-	80.8
83.3	88.1	93.6	97.4
99.6	99.6	-	102.6
112.4	123.4	124.4	126.4
-	-	133.4	-
-	-	-	-
-	192.3	192.6	

f) having a molecular formula: $C_{54}H_{84}N_3O_{22}S_4I$; and

g) having a molecular weight:1381.

12. A compound LL-E33288β_2-I having the following R_f values in the indicated solvent systems on TLC on silica gel sheets:

a) ethyl acetate saturated with 0.1M aqueous potassium dihydrogen phosphate, R_f=0.32.

b) 3% isopropanol in ethyl acetate saturated with 0.1M aqueous potassium dihydrogen phosphate, R_f=0.41; and

c) ethyl acetate:methanol (95:5), R_f=0.45.

13. A compound LL-E33288γ_1-I

a) having the following R_f values in the indicated solvent systems on TLC on silica gel sheets:

i) ethyl acetate saturated with 0.1M aqueous potassium dihydrogen phosphate, R_f = 0.18;

ii) 3% isopropanol in ethyl acetate saturated with 0.1M aqueous potassium dihydrogen phosphate, R_f = 0.28; and

iii) ethyl acetate: methanol (95:5), R_f = 0.27;

b) containing only the following elements: C, H, N, O, S and I;

c) having an approximate elemental analysis: C 48.8; H 5.4; N 2.8; S 9.0; and I 9.2;

d) having a molecular weight: 1367;

e) having a molecular formula: C_{53} H_{82} N_3 O_{22} S_4 I;

f) having an ultraviolet absorption spectra as shown in Figure XV of the drawings;

g) having an infrared absorption spectrum as shown in Figure XVI of the drawings;

h) having a proton magnetic resonance spectrum as shown in Figure XVII of the drawings; and

i) having a carbon 13 magnetic resonance spectrum as shown in Figure XVIII of the drawings, significant peaks as listed below:

14.5(q)	17.6(q)	17.6(q)	18.9(q)
-	-	22.8(q)	-
25.4(q)	34.1(t)	37.0(t)	39.1(t)
42.3(t/s)	-	51.5(d)	52.8(q)
54.8(t)	56.3(q)	57.2(q)	-
60.4(d)	60.9(q)	61.3(t)	61.7(q)
67.0(d)	68.4(d)	68.5(d)	69.2(d)
69.7(d)	70.5(d)	71.1(d)	71.8(d)
72.1(s)	75.7(d)	75.8(d)	80.9(d)
82.8(s)	88.1(s)	93.5(s)	97.3(d)
99.6(d)	99.7(d)	100.8(s)	102.6(d)
-	123.4(d)	124.4(d)	126.2(d)
130.2(s)	131.0(s)	133.4(s)	139.1(s)
143.0(s)	145.1	150.6(s)	151.5(s)
154.5	192.0(s)	192.5(s)	

14.) A compound LL-33288δ_1-I having the following R_f values in the indicated solvent systems on TLC on silica gel sheets:

a) ethyl acetate saturated with 0.1M aqueous potassium dihydrogen phosphate, R_f = 0.11; and

b) 3% isopropanol in ethyl acetate saturated with 0.1M aqueous potassium dihydrogen phosphate, R_f = 0.19.

15. A process for producing antibiotics LL-E33288α_1-Br; LL-E33288α_2-Br; LL-E33288α_3-Br; LL-E33288α_4-Br; LL-E33288β_1-Br; LL-E33288β_2-Br and LL-E33288γ_1-Br which comprises aerobically fermenting the organism <u>Micromonospora</u> <u>echinospora</u> ssp. <u>calichensis</u> NRRL 15839 or mutants thereof including NRRL 15975 in a liquid medium containing assimilable sources of carbon, nitrogen,

bromine and inorganic salts, until substantial antibiotic activity is imparted to said medium and then recovering the antibiotics therefrom.

16. A process for producing antibiotics LL-E33288α_1-Br; LL-E33288α_2-Br; LL-E33288α_3-Br; LL-E33288α_4-Br; LL-E33288β_1-Br; LL-E33288β_2-Br; and LL-E33288γ_1-Br which comprises aerobically fermenting a liquid medium containing assimilable sources of carbon, nitrogen, bromine and inorganic salts; which medium has been inoculated with a viable culture of the organism Micromonospora echinospora ssp. calichensis NRRL 15839 or mutants thereof including NRRL 15975, maintaining said fermentation culture at a temperature of about 24-32°C for a period of approximately 90-200 hours, harvesting the mash and extracting the antibiotics.

17 A process for producing antibiotics LL-E33288α_1-I; LL-E33288α_2-I; LL-E33288α_3-I; LL-E33288β_1-I; LL-E33288β_2-I; LL-E33288γ_1-I; and LL-E33288δ_1-I which comprises aerobically fermenting the organism Micromonospora echniospora ssp. calichensis NRRL 15839 or mutants thereof including NRRL 15975 in a liquid medium containing assimilable sources of carbon, nitrogen, iodine and inorganic salts, until substantial antibiotic activity is imparted to said medium and then recovering the antibiotics therefrom.

18. A process for producing antibiotics LL-E33288α_1-I; LL-E33288α_2-I; LL-E33288α_3-I; LL-E33288β_1-I; LL-E33288β_2-I; LL-E33288γ_1-I; and LL-E33288δ_1-I which comprises aerobically fermenting a liquid medium containing assimilable sources of carbon, nitrogen, iodine and inorganic salts, which medium has been inoculated with a viable culture of the microorganism Micromonospora echinospora ssp. calichensis NRRL 15839 or mutants thereof including NRRL 15975, maintaining said fermentation culture at a temperature of about 24-32°C for a period of approximately 90-200 hours, harvesting the mash and extracting the antibiotics.

19 . A culture containing the microorganism

Micromonospora _echinospora_ ssp. _calichensis_, NRRL 15839, said culture being capable of producing the LL-E33288 complex in recoverable quantity upon aerobic fermentation in an aqueous medium containing assimilable sources of carbon nitrogen, inorganic salts, and either iodine or bromine or both.

20. A culture containing the microorganism _Micromonospora_ _echinospora_ ssp. _calichensis_ NRRL 15975, said culture being capable of producing the LL-E33288 complex in recoverable quantity upon aerobic fermentation in an aqueous medium containing assimilable sources of carbon, nitrogen, inorganic salts, and either iodine or bromine or both.

21. A process for producing the antibiotics referred to in Claim 15, substantially as herein described and illustrated.

DATED THIS 15 DAY OF NOVEMBER 1985

J.R. Steyn

ADAMS & ADAMS

APPLICANTS PATENT ATTORNEYS

18

SHEET No. 5

ORIGINAL

ULTRAVIOLET ABSORPTION SPECTRA OF LL-E33288γ
20 μg/ml SOLUTION

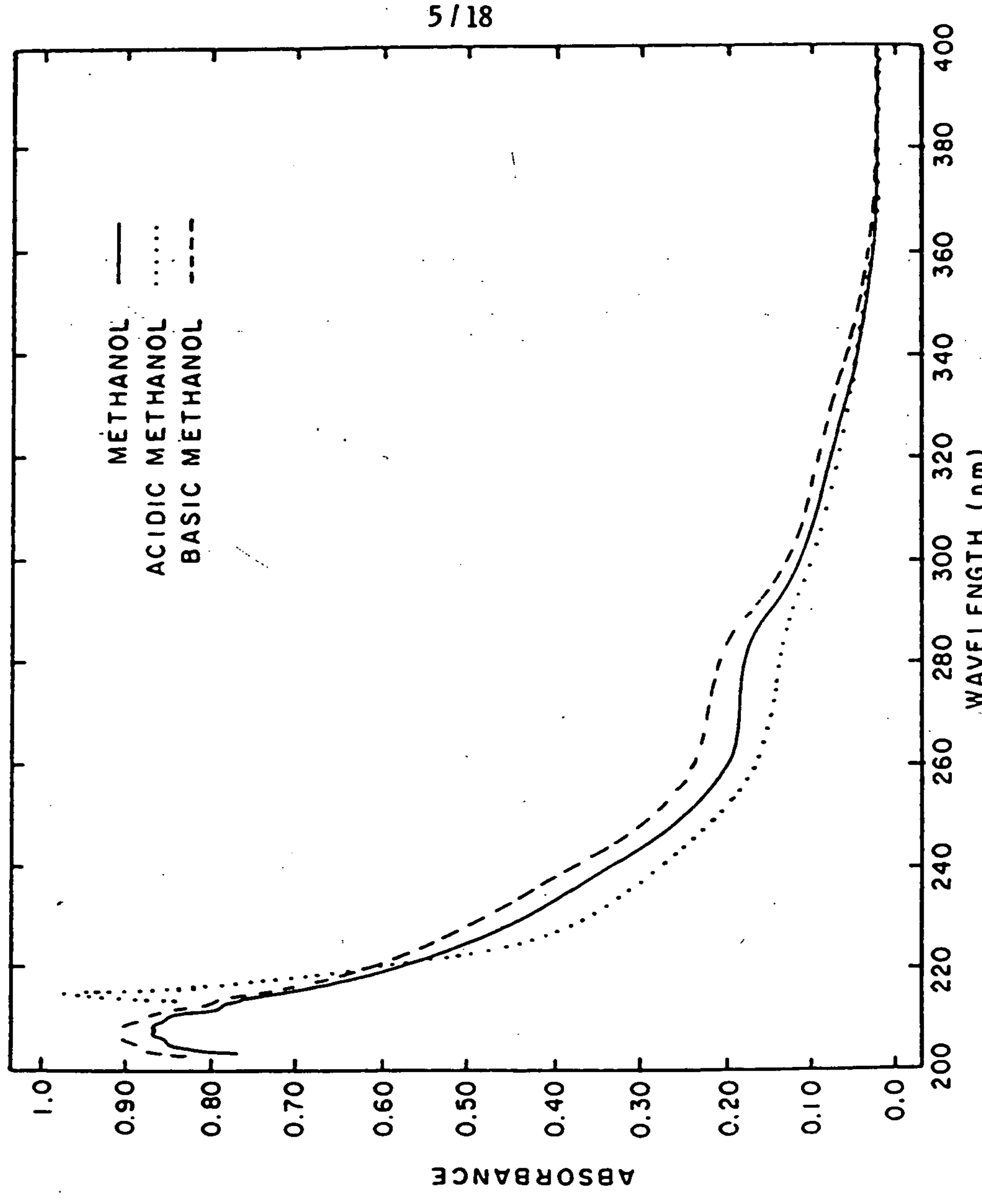

FIGURE V

ADAMS & ADAMS
APPLICANTS PATENT ATTORNEYS

18 SHEET (6)
SHEET No. 6
ORIGINAL

6/18

INFRARED ABSORPTION SPECTRUM OF LL-E33288 γ

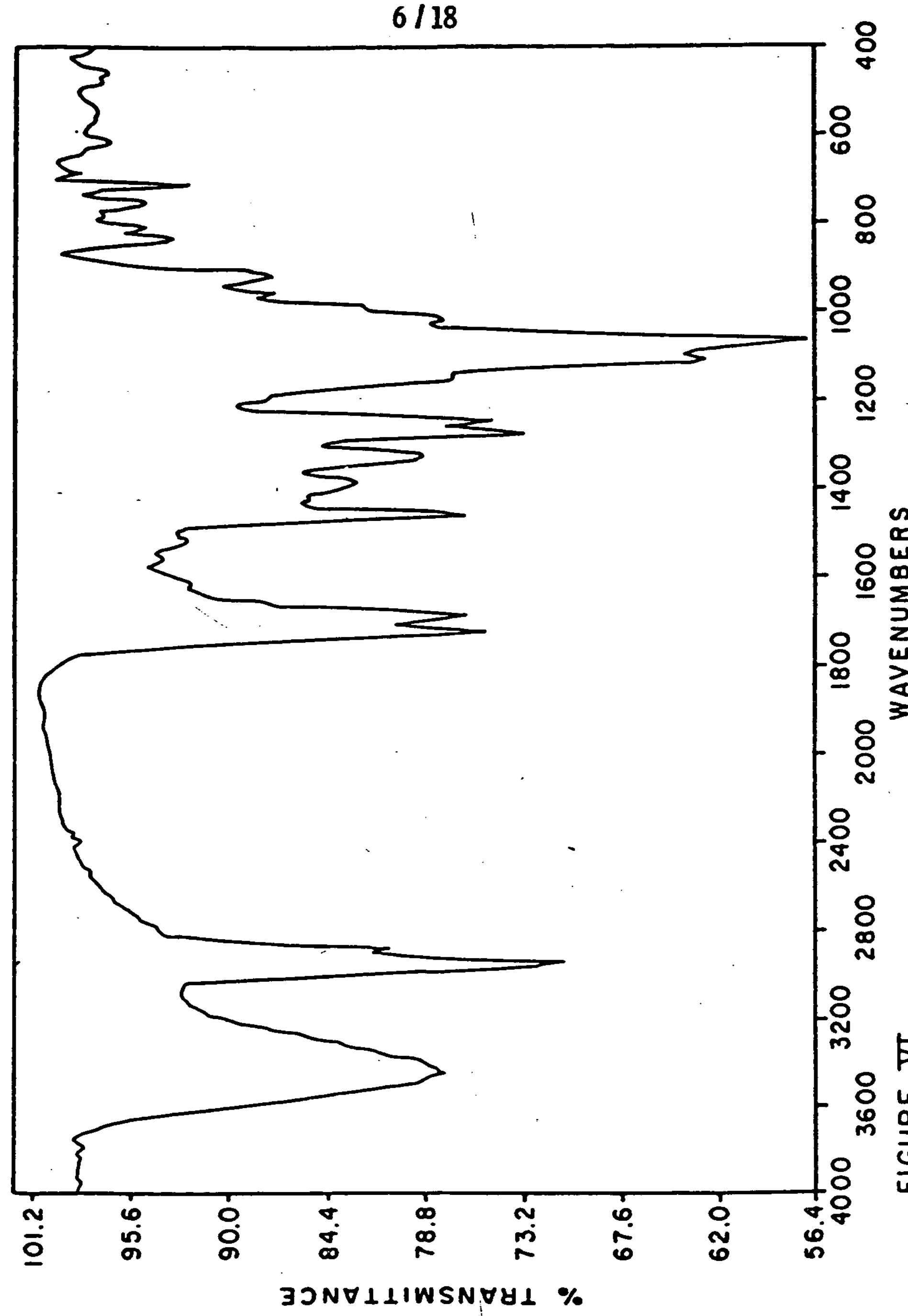

FIGURE VI

ADAMS & ADAMS
APPLICANTS PATENT ATTORNEYS

PROTON MAGNETIC RESONANCE SPECTRUM OF
LL-E33288γ

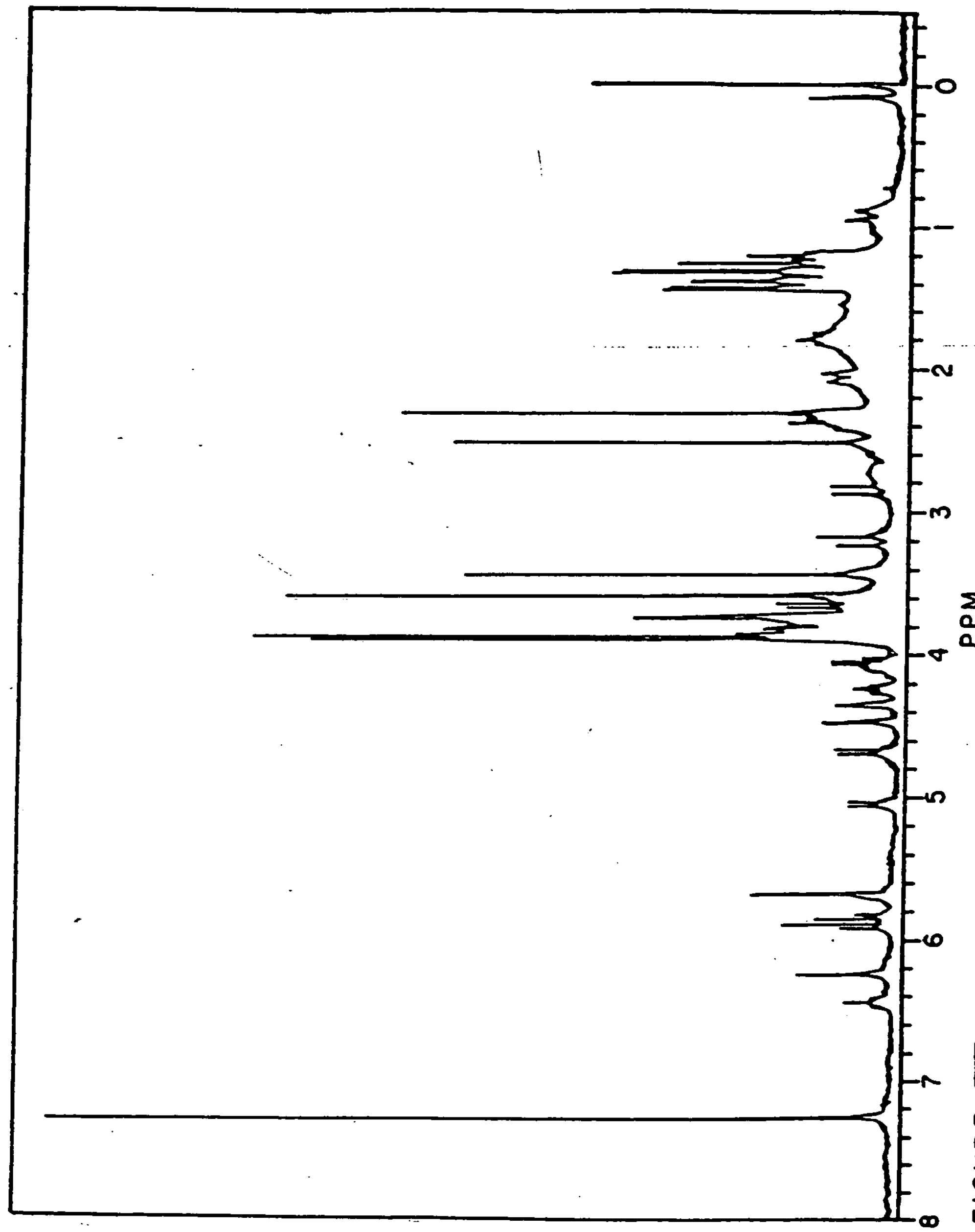

FIGURE VII

18 SHEET (S)
SHEET No. 8
ORIGINAL

13CNMR OF LL-E33288 γ_1 Br

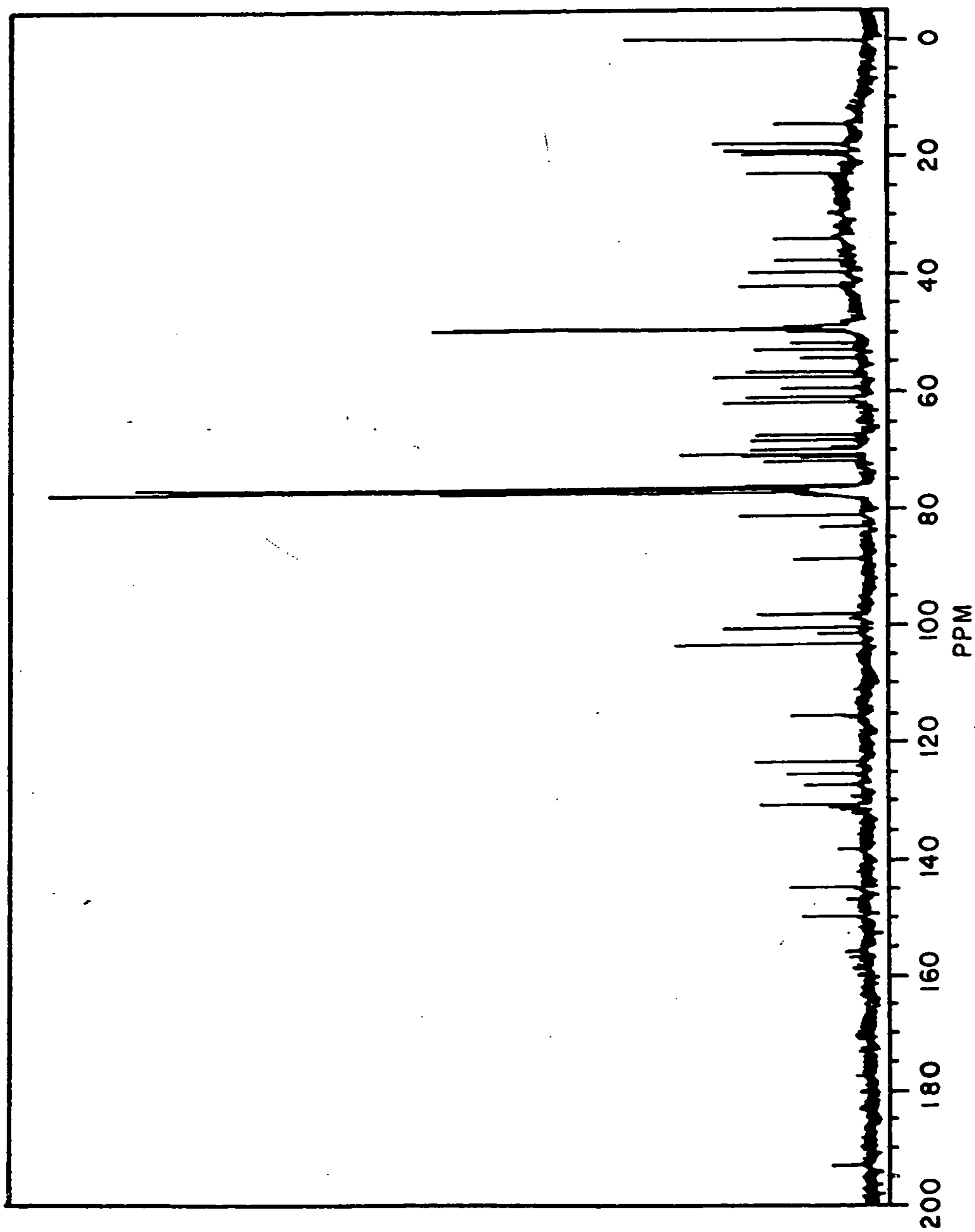

FIGURE VIII

Adams & Adams
APPLICANTS PATENT ATTORNEYS

18 SHEET (S)
SHEET No. 9
ORIGINAL

PMR OF LL-E 33288 α_2 I

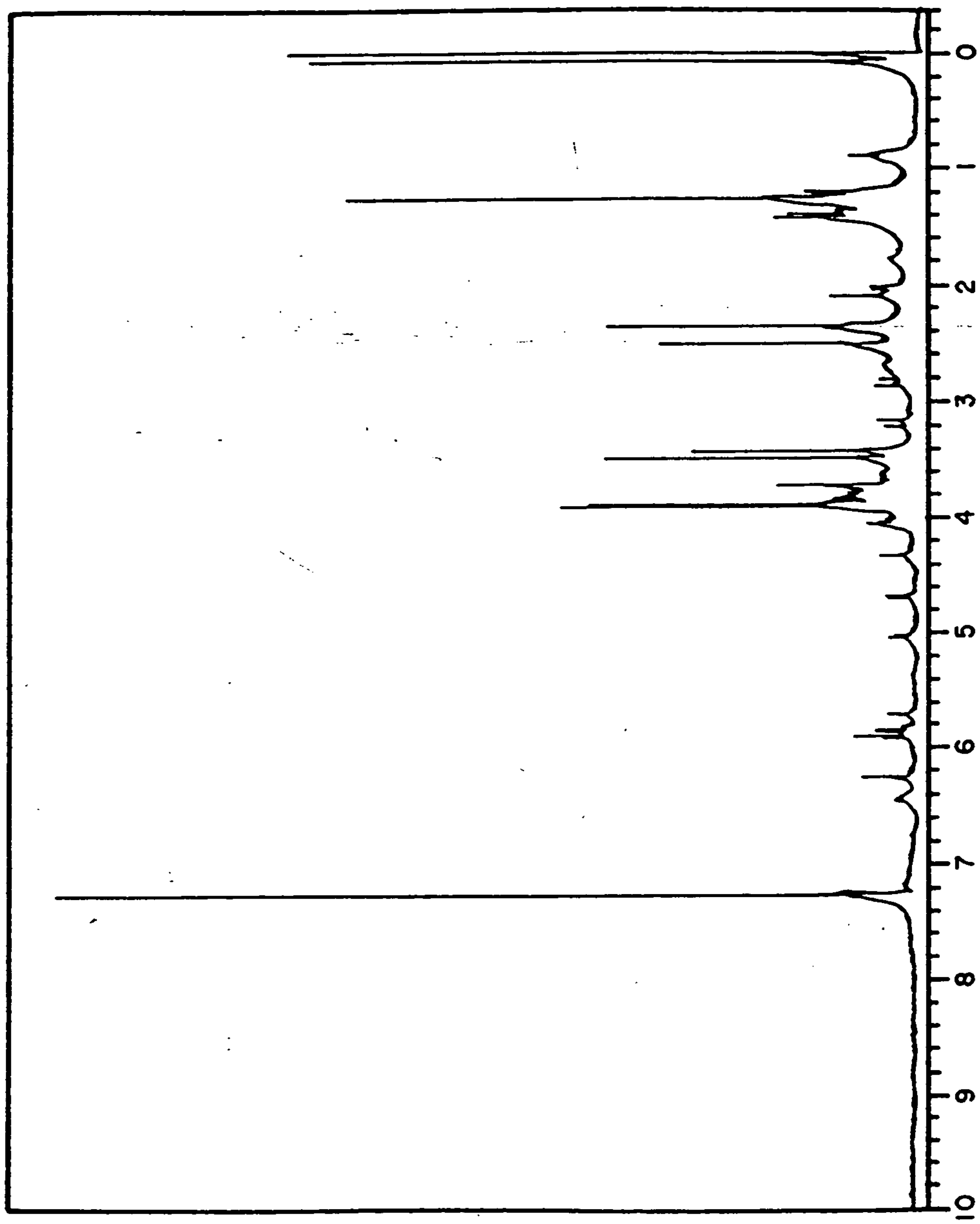

FIGURE IX

ADAMS & ADAMS
APPLICANTS PATENT ATTORNEYS

18 SHEET (S)
SHEET No. 10
ORIGINAL

PMR OF LL-E33288α_3 I

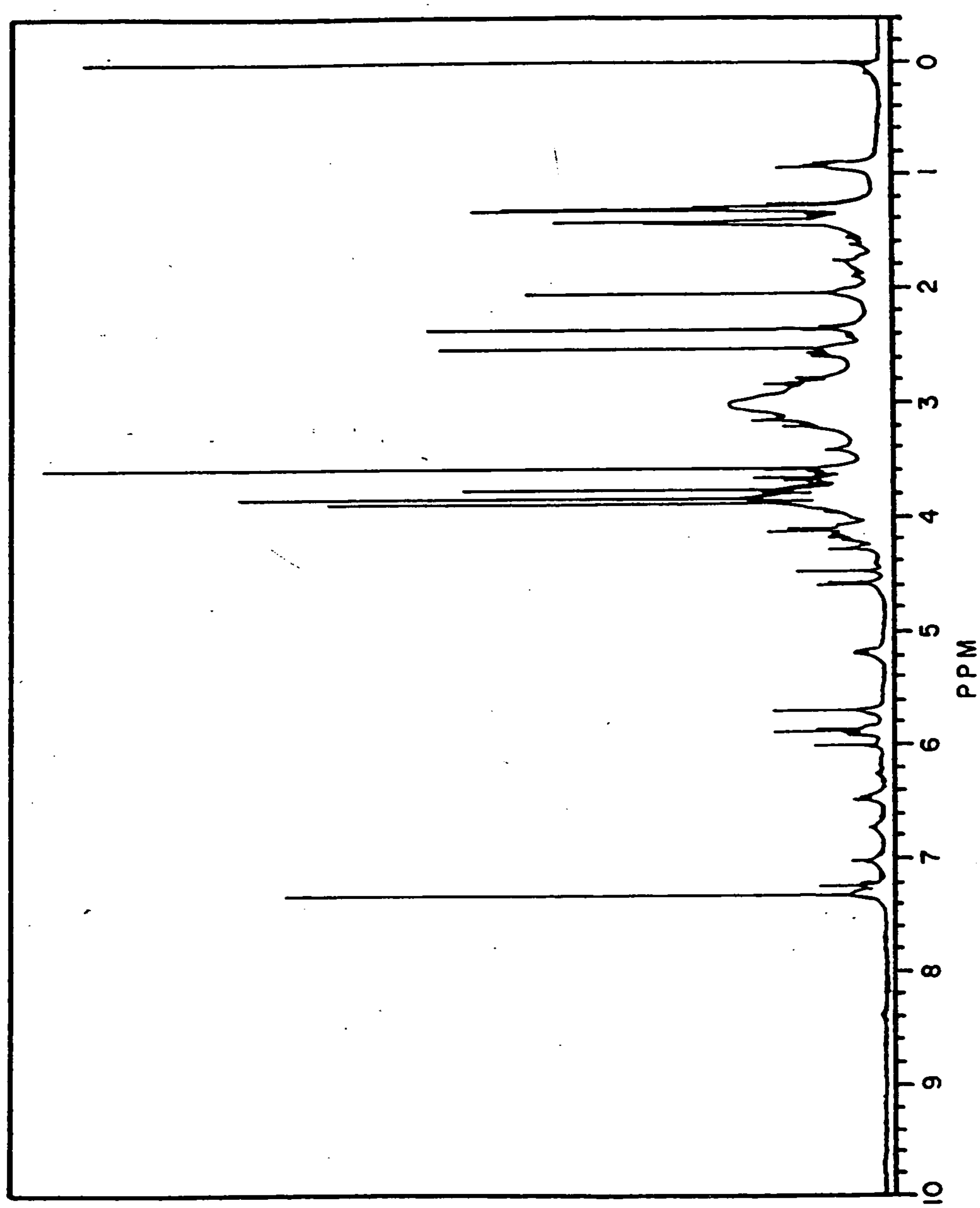

FIGURE X

ADAMS & ADAMS
APPLICANTS PATENT ATTORNEYS

18 SHEET (S)
SHEET No. 11
ORIGINAL

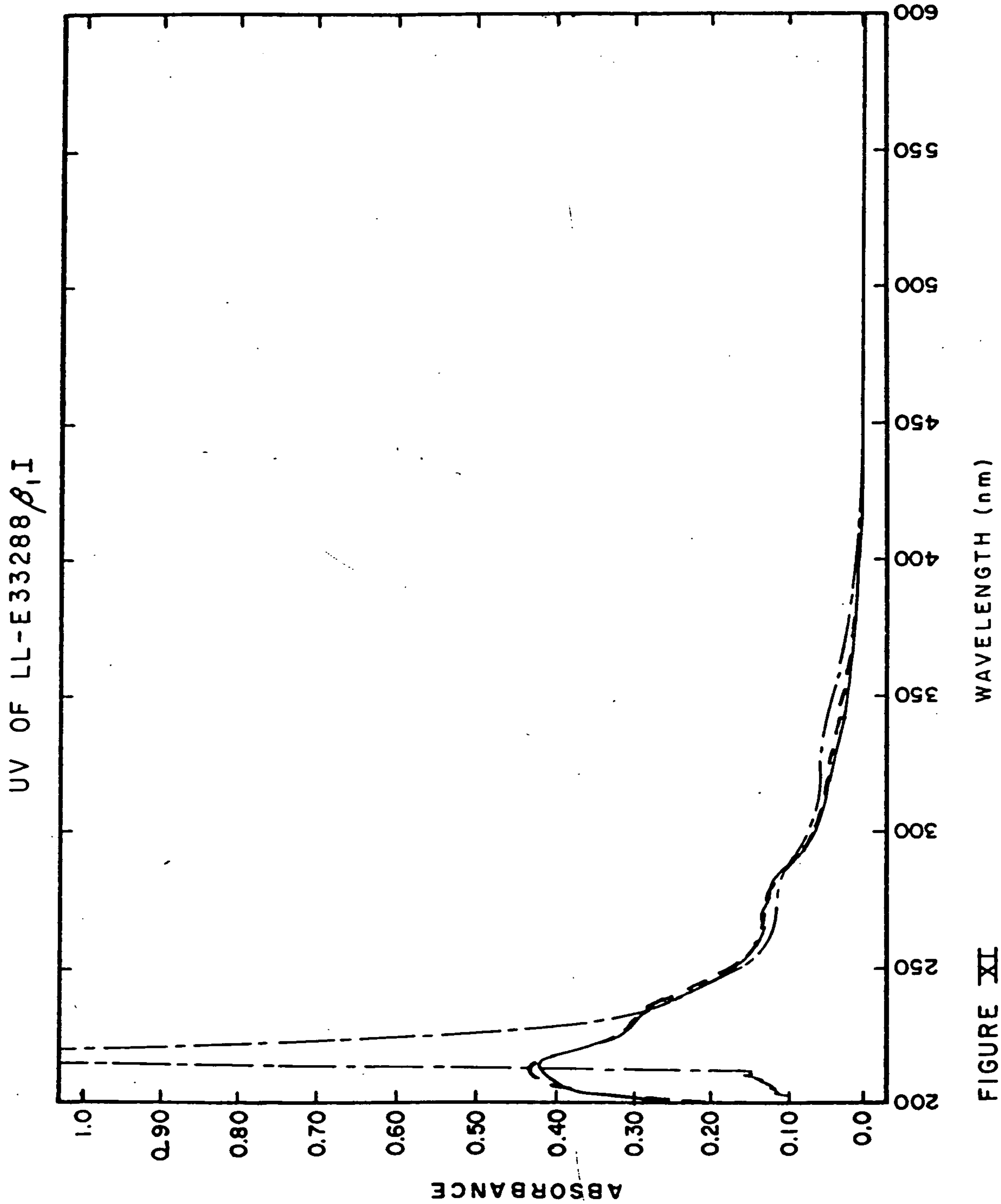

FIGURE XI

ADAMS & ADAMS
APPLICANTS PATENT ATTORNEYS

AMERICAN CYANAMID COMPANY - APPL. NO. 85/8794

18 SHEET (S)
SHEET No. 12
ORIGINAL

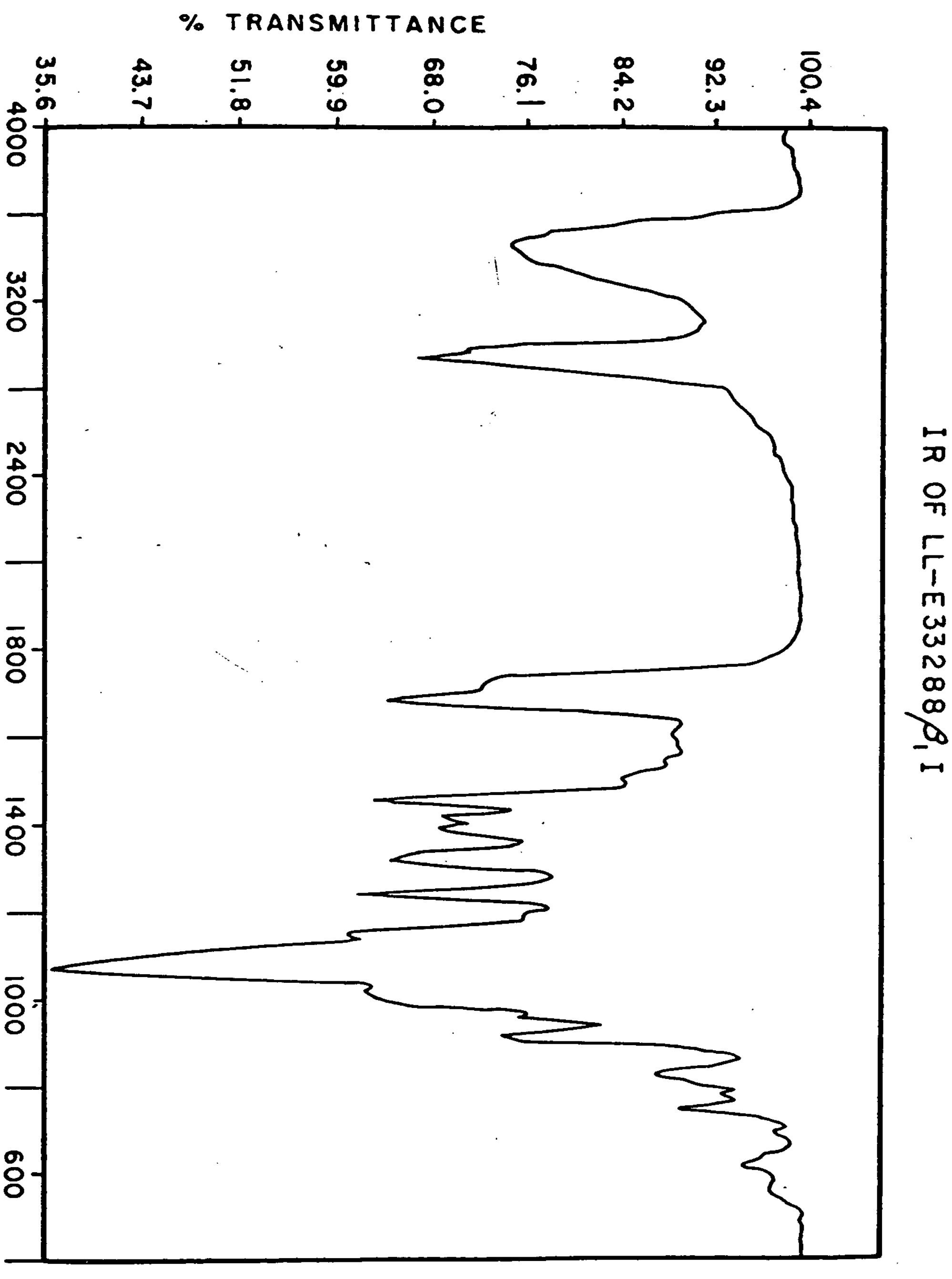

FIGURE XII

12/18

ADAMS & ADAMS
APPLICANTS PATENT ATTORNEYS

18 SHEET (S)
SHEET No. 13
ORIGINAL

PMR OF LL-E33288β_1I

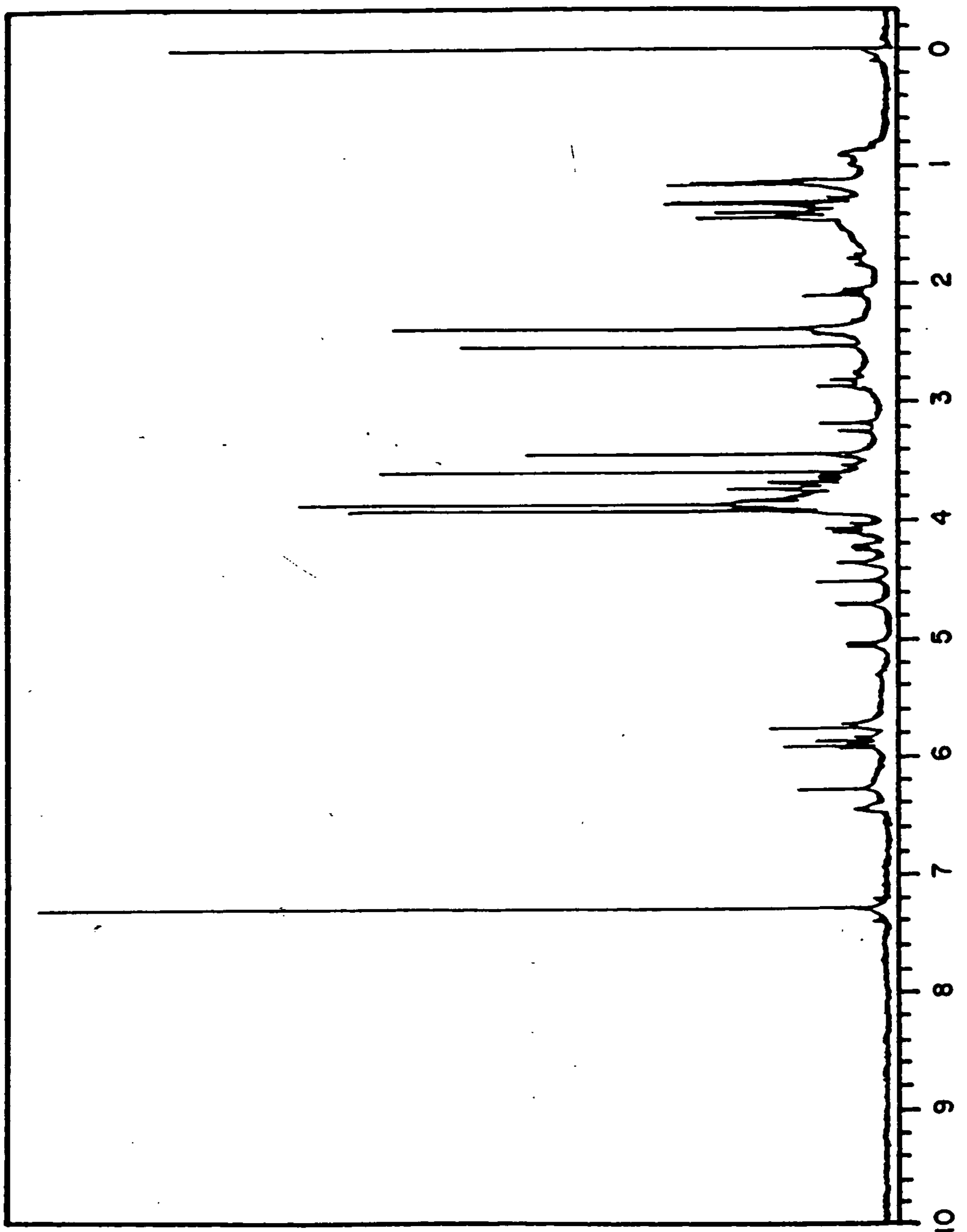

FIGURE XIII

ADAMS & ADAMS
APPLICANTS' PATENT ATTORNEYS

AMERICAN CYANAMID COMPANY - APPL. NO. 85/8794

18 SHEET (S)
SHEET No. 14
ORIG

13CNMR OF LL-E33288β_1I

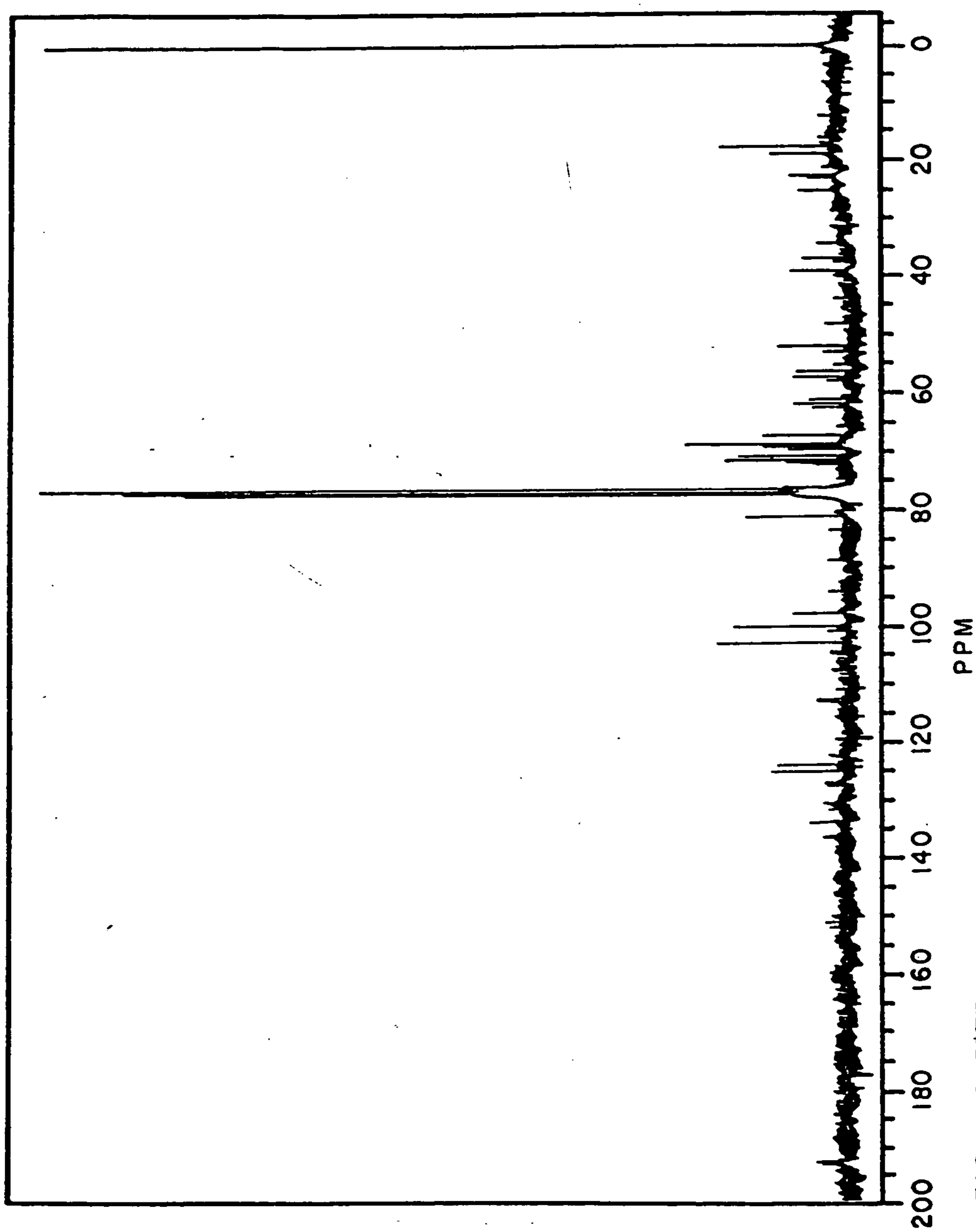

FIGURE XIV

ADAMS & ADAMS
APPLICANTS PATENT ATTORNEYS

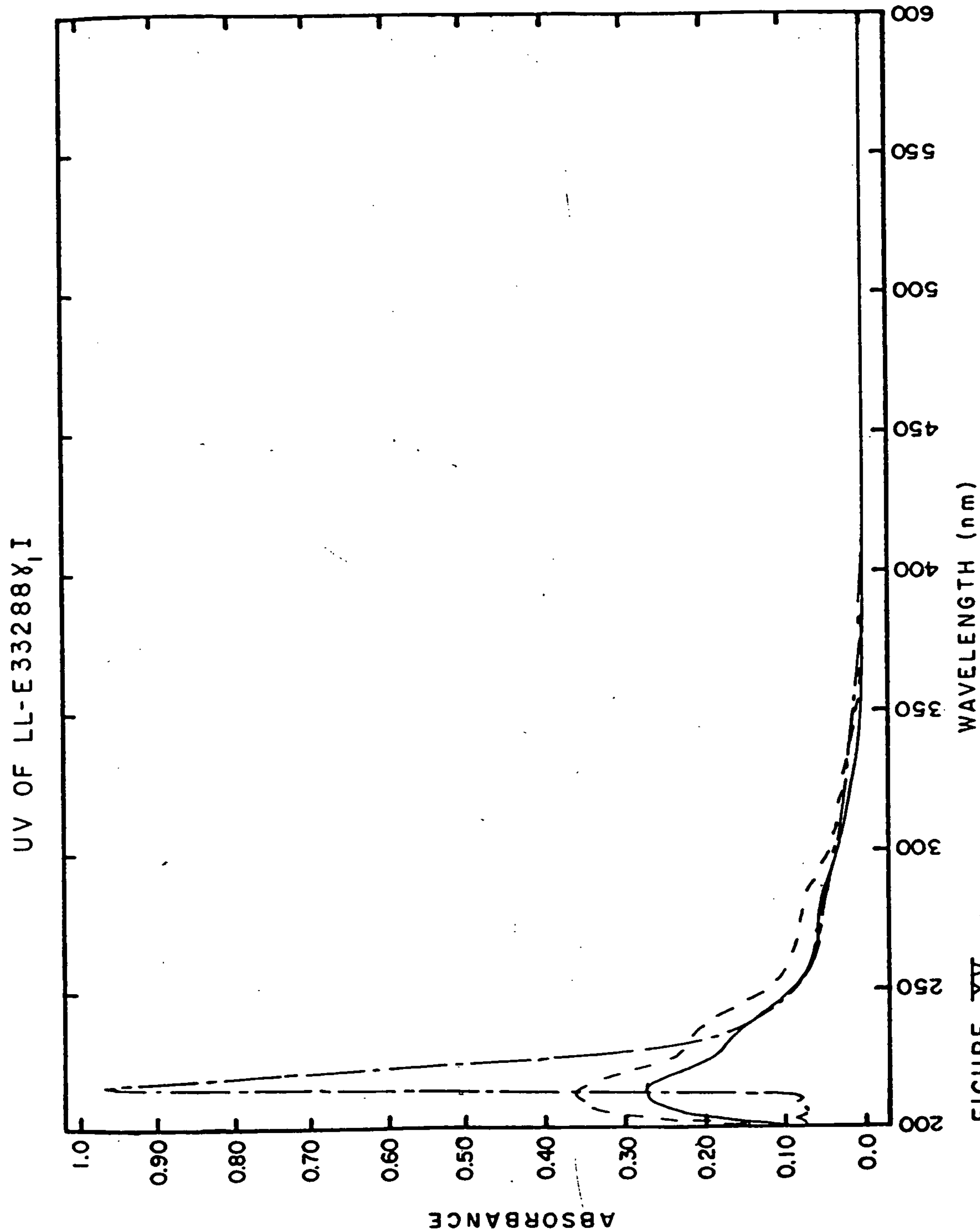

FIGURE XV

ADAMS & ADAMS
APPLICANTS PATENT ATTORNEYS

18 SHEET (S)
SHEET No. 16
ORIGINAL

IR OF LL-E33288γ_1 I

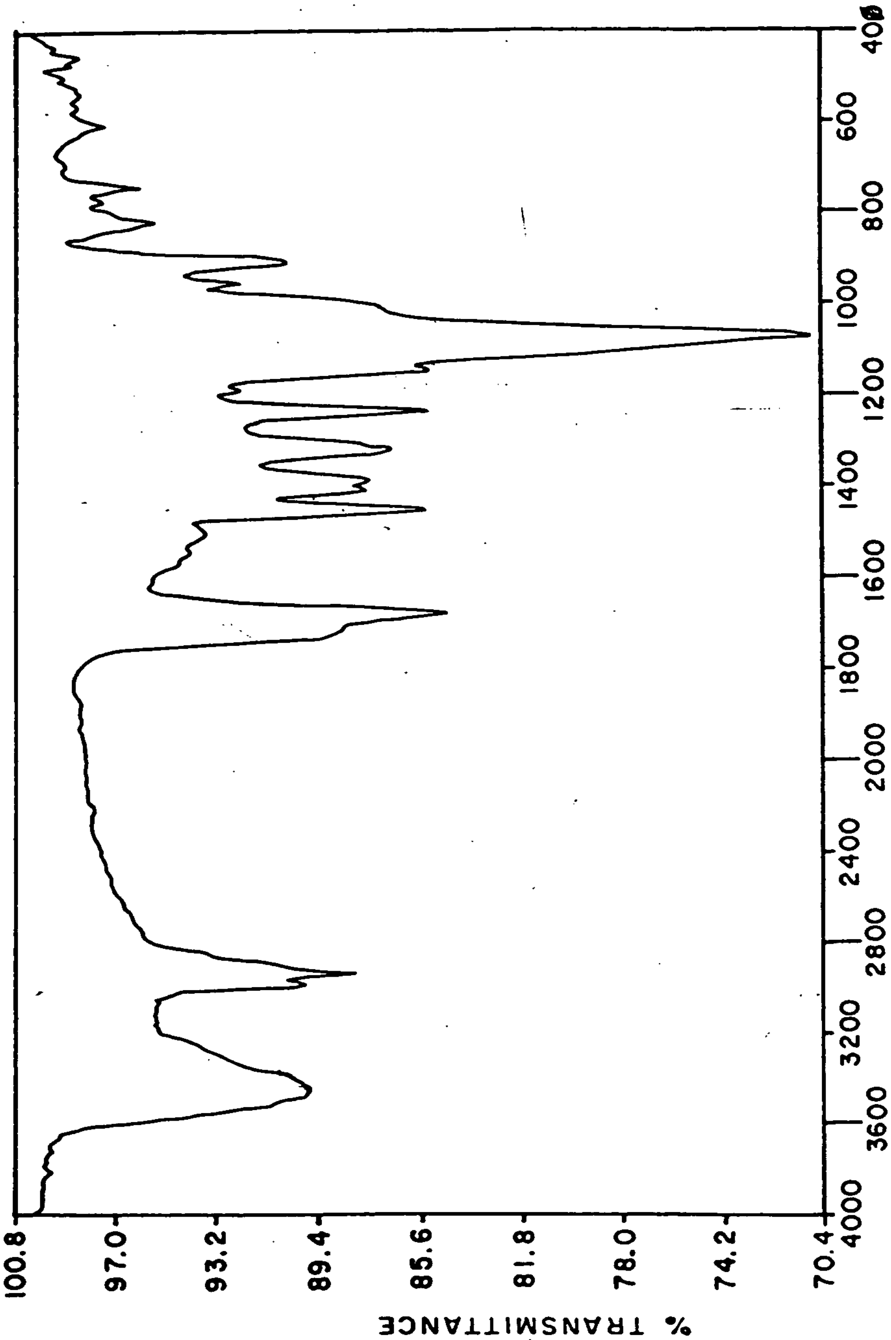

FIGURE XVI

ADAMS & ADAMS
APPLICANTS PATENT ATTORNEYS

18 SHEET (S)
SHEET No 17
ORIGI

PMR OF LL-E33288γ_1 I

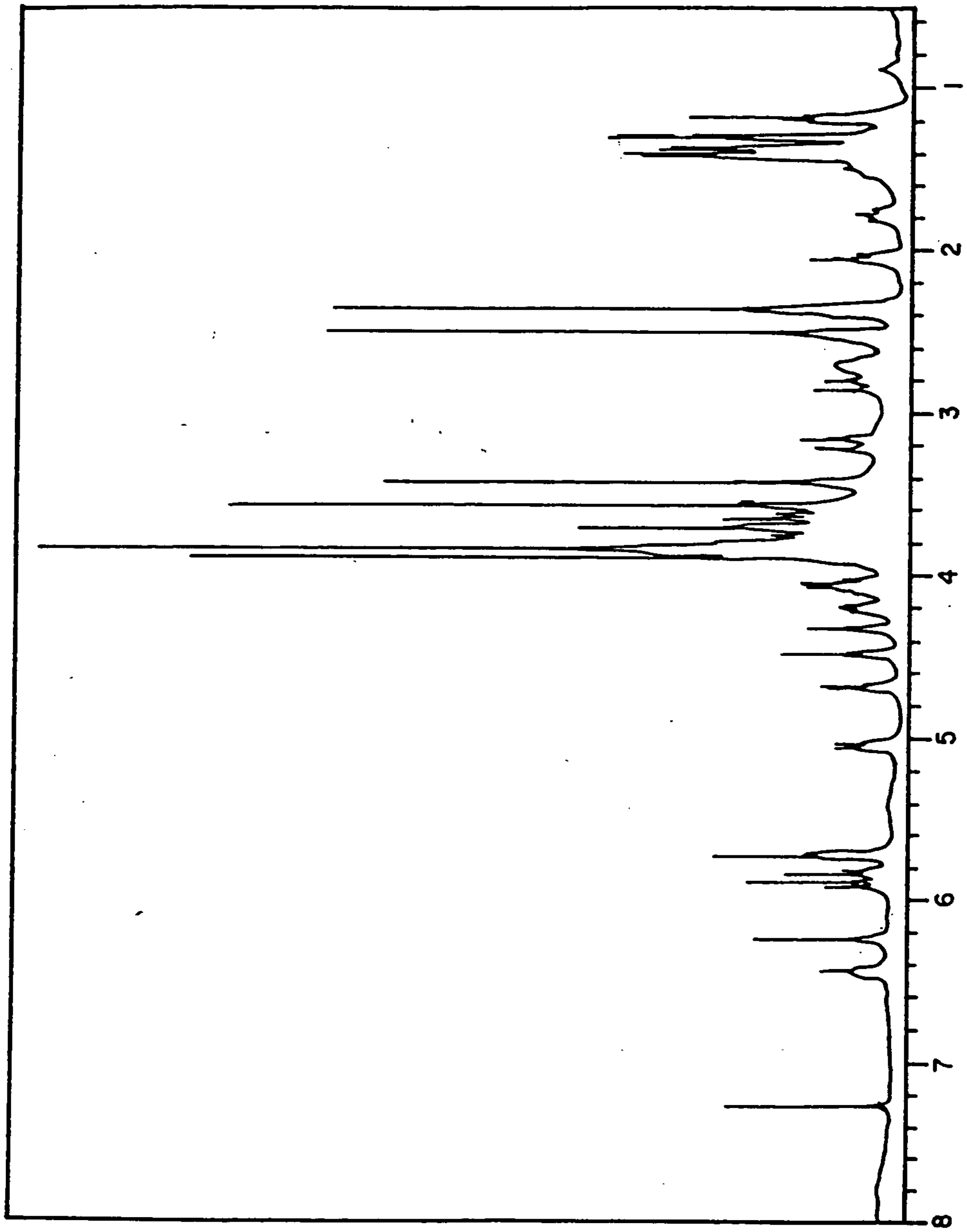

FIGURE XVII

APPLICANTS PATENT ATTORNEYS

18 SHEET (S)
SHEET No. 18
ORIGIN

13CNMR OF LL-E33288γ_1I

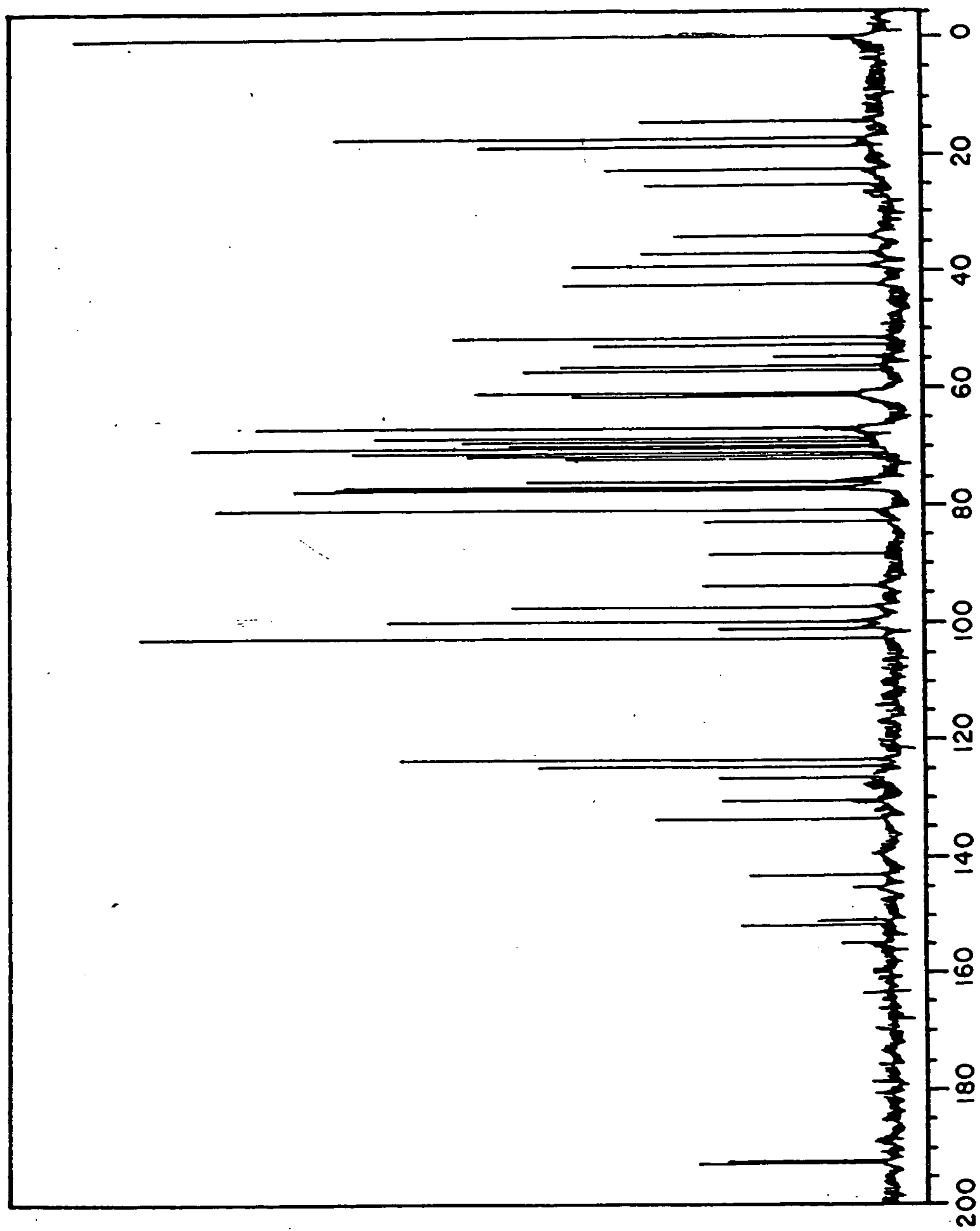

FIGURE XVIII

APPLICANTS PATENT ATTORN